CHARACTERIZATION OF INTRASPECIFIC VARIATIONS OF *BELONOLAIMUS LONGICAUDATUS* BY MORPHOLOGY, DEVELOPMENTAL BIOLOGY, HOST SPECIFICITY, AND SEQUENCE ANALYSIS OF ITS-1 rDNA

By

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To my husband, Sung-Ho Park, and my lovely son, Ho-Jin Park

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Major Department: Entomology and Nematology

Sting nematode, *Belonolaimus longicaudatus*, is one of the most economically important plant-parasitic nematodes in the southeastern United States. Our objectives were to determine the intraspecific variation based on morphology, developmental biology, host specificity, and ITS-1 DNA sequence among isolates collected from different geographical locations and host crops. Five isolates that were compared by all criteria included: HA isolate from potato in Hastings, FL; GV isolate from bermudagrass in Gainesville, FL; LA isolate from citrus in Lake Alfred, FL; GA isolate from cotton in Tifton, GA; and NC isolate from corn in Scotland County, NC. Two additional isolates from corn (NB) and bermudagrass (TX) were obtained from Columbus, NB and Poteet, TX, and compared with the other five isolates with respect to ITS-1 DNA sequence. The ITS-1 DNA sequence of a South Carolina (SC) isolate was downloaded from the Gene Bank and added for sequence comparison.

Females of the LA and NC isolates were larger in body length and tail length and the LA isolate had a longer stylet length compared with the other isolates ($P \leq 0.05$). The GV isolate had the shortest stylet length and body length among all isolates ($P \leq 0.05$). Development from egg to adult, which was observed on excised corn root in Gamborg B-5 medium at 28 °C, ranged from 18.1 days (GA isolate) to 25 days (NC isolates). The GA and LA isolates reproduced on five crop plants tested, whereas the NC and GV isolates reproduced poorly on all plants except cotton. The LA (citrus) and HA (potato) isolates reproduced better on their original hosts than on other plants tested. However, the GV (bermudagrass) isolate did not reproduce well on its original host. The length of the ITS-1 region were identical, 468 bp for all isolates, excepting the TX isolate which had a length of 427 bp with a deletion of 41 bp. Sequences of the ITS-1, determined in two separate PCR-generated clones for each isolate, showed some differences in a single nucleotide position. Significant patterns of heterogeneity were seen in ITS-1 sequences from different isolates. Based on three different phylogenetic analyses of ITS-1, the TX, NB, and SC isolates were closely related, but completely different from the HA, LA, GV, GA, and NC isolates.

CHAPTER 1
GENERAL INTRODUCTION

Sting nematodes (*Belonolaimus* spp.) are distributed in most of the Atlantic, and Gulf coastal regions of Florida, Georgia, South Carolina, Virginia, Alabama, Mississippi (Christie et al., 1952; Graham and Holdeman, 1953; Holdeman, 1955; Owens, 1951), New Jersey (Hutchinson and Reed, 1956; Myers, 1979), and in some regions in the middle of the United States including Arkansas (Riggs, 1956), Kansas (Dickerson et al., 1972), and Texas (Christie, 1959; Norton, 1959; Wheeler and Starr, 1987). They are also reported from Nebraska (Kerr and Wysong, 1979) and were recently introduced into California (Mundo-Ocampo et al., 1994). Outside the United States, sting nematodes have been reported from Brazil (Monterio and Lordello, 1977), Costa Rica (Lopez, 1978), Puerto Rico (Roman, 1964), Bahamas and Bermuda (Perry and Rhoades, 1982), and Australia (Siviour and Mcleod, 1979).

There are currently nine species of *Belonolaimus* recognized worldwide, *B. gracilis*, *B. longicaudatus*, *B. euthychilus*, *B. maritimus, B. nortoni, B. anama*, *B. jara*, *B. lineatus*, and *B. lolii*. In the southeastern United States the most common species encountered in agriculture is *B. longicaudatus*, Rau (1958). This species was considered among the most important plant-parasitic nematodes in the southeastern United States after Christie et al. (1952) established its pathogenicity on strawberry, celery, and sweet corn in central Florida. The geographical distribution of *B. longicaudatus* is limited to certain geographical regions that have high sand content. The nematode is associated with a wide variety of host plants that includes vegetables, grains, fruits, forage crops,

turfgrasses, ornamentals, and forest trees (Christie, 1953; Esser, 1976; Holdeman, 1955; Robbins and Barker, 1973).

Pathogenicity tests of *B. longicaudatus* have been done on many other crops including peanut (Good, 1968; Owens, 1951; Perry and Norden, 1963), cotton (Graham and Holdeman, 1953) strawberry, celery, sweet corn (Christie, 1952), collard, kale, cauliflower (Khuong, 1974), and citrus (Standifer and Perry, 1960; Suit and DuCharme, 1953). These studies proved the significance of *B. longicaudatus* in agriculture. There have been several conflicting reports, regarding the pathogenicity, host range, and morphology within species of sting nematodes from different geographical regions or hosts (Abu-Gharbieh and Perry, 1970; Owens, 1951; Perry and Norden, 1963; Robbins and Barker, 1973; Robbins and Hirschmann, 1974), which suggests the possible existence of physiological races of *B. longicaudatus*.

Accurate identification of plant-parasitic nematodes is paramount for their effective control and management. Sometimes morphological character alone will not enable us to identify physiologically different populations of *B. longicaudatus*. This may lead to the confusing of pathogenically different, but morphologically similar species (Abad, 1994). For instance, the variation in host range among different isolates of *B. longicaudatus* may cause confusion in the designation of a successful management program, especially in setting a cropping system for nematode management. Effective management strategies cannot be developed without reliable identification.

To help identify nematodes, especially closely related nematode groups among species or races, many nematologists are adapting advanced technological methods. Until the 1990s, the biochemical methods used were mostly protein analysis (Dickson et

al., 1970; Dickson et al., 1971; Esbenshade and Triantaphyllou, 1985; Ferris et al., 1987; Huettel et al., 1983a; Huettel et al., 1983b; Hussey and Krusberg; 1972; Payan and Dickson, 1990; Pozdol and Noel, 1984; Trudgill and Parrott, 1972). In late 1980s, nucleic acid analysis was introduced in nematology, which activated more research involving molecular biology (Bolla et al., 1987; Bolla et al., 1988; Curran et al., 1986; Hyman, 1988; Kalinski and Huettel, 1988; Powers et al., 1986; Powers and Sandall, 1988; Radice et al., 1988). These biochemical techniques were useful to the application of nematode taxonomy and systematics, especially among closely related taxa.

Polymerase chain reaction (PCR) (Saikai, 1989) was developed as a relatively convenient and powerful tool for amplification of selected regions of chromosomal and extrachromosomal DNA. The development of PCR accelerated the progress of molecular biology in nematology. The combinations of PCR-RFLP (restriction fragment length polymorphism) (Bekal et al., 1997; Cherry et al., 1997; Fallas et al., 1996; Gasse and Hoste, 1995; Hiatt et al., 1995; Hoyer et al., 1998; Newton et al., 1998; Orui, 1996; Orui, 1997; Subbotin et al., 2000; Vrain et al., 1992; Waeyenberge et al., 2000; Wendt et al., 1995; Zijlstra, 1997; Zijlstra et al., 1995), PCR-RAPD (random amplified polymorphic DNA) (Esquibet et al., 1998; Jobet et al., 1998; Thiery et al., 1997), and sequencing of target genes amplified by PCR (Adams et al, 1998; Ferris et al., 1993; Ferris et al., 1994; Ferris et al., 1995; Ferris et al., 1999; Zhu et al., 2000) were used to detect and differentiate closely related taxa of nematodes.

The ITS (Internal Transcribed Spacer) region in ribosomal DNA array was shown to have sequence variability greater than that observed for the region encoding the 5.8S, 18S, and 28S sequences destined for the mature ribosome. Thus, sequence variability in

ITS-1 provides a measure for the determination of phylogenetic differences among eukaryotes that were designated as a species. In nematology, ITS was evaluated as a taxonomic marker for nematodes through a diverse nematode survey (Powers et al., 1997). The ITS is a non-coding region located between functional coding genes. The usefulness of this genetic marker to differentiate among closely related taxa has been proven in plants, insects, and animal parasitic nematodes (Adams et al, 1998; Bekal et al., 1997; Cherry et al., 1997; Ferris et al., 1999; Gasse and Hoste, 1995; Hoyer et al., 1998; Newton et al., 1998; Orui, 1997; Subbotin et al., 2000; Vrain et al., 1992; Waeyenberge et al., 2000; Zijlstra et al., 1995; Zhu et al., 2000).

The taxonomy of *B. longicaudatus* has been an issue for approximately 50 years. The level of variation in morphology, host pathogenicity, and host range of *B. longicaudatus* suggested the existence of additional species or physiological races. In previous studies, however, the variations were compared among North Carolina and Georgia isolates or among Florida isolates, and were approached only by traditional taxonomic characteristics. In this study, the numbers of isolate were increased including Florida, Georgia, and North Carolina sources and were compared by both traditional and molecular methods. Additional isolates from Texas, Nebraska, and South Carolina also were used for molecular analyses. Furthermore, the biology of *B. longicaudatus* was observed *in vitro* and the developmental time of life stages was compared among different isolates. Therefore, the objectives of this work were to compare variation levels in biology, morphology, and host specificity among isolates of *B. longicaudatus* from different geographical locations and hosts; to determine whether there are significant variations in the sequence of ITS-1 in rDNA among different isolates of *B.*

longicaudatus; and to examine the use of ITS-1 of rDNA as a genetic marker to differentiate *B. longicaudatus* isolates based on phylogenetic analysis. Finally, new data on variation will help in the understanding of characteristics of each isolate and the relationship among different isolates based on characters studied.

CHAPTER 2
REVIEW OF LITERATURE

Taxonomy

The sting nematode, *Belonolaimus longicaudatus* (Rau, 1958), has been recognized as an important plant ectoparasitic nematode since the late 1940s. The first reported species was *Belonolaimus gracilis* (Steiner, 1942), which was identified from soil collected around roots of slash and longleaf pines in nurseries near Ocala, Florida. A few years later, however, Rau reported that *B. gracilis* was a rare species, and that it had not been observed since its original description (Rau, 1958).

Rau reported a new species of *Belonolaimus*, which was morphologically similar, but differed from *B. gracilis* in that it had a longer tail and shorter stylet length. Therefore, he named the nematode *B. longicaudatus*. Unlike *B. gracilis*, *B. longicaudatus* was found on many different hosts from various places in Florida, and it is currently recognized as the most common sting nematode in the United States (Rau, 1958; 1961).

In 1963, Rau described three more species: *B. euthychilus*, *B. maritimus*, and *B. nortoni*. Other species, *B. hastulatus* from Queensland, Australia (Colbran, 1960), *B. lineatus* from Puerto Rico (Roman, 1964), and *B. lolii* from Australia (Siviour, 1978) also were described. *Belonolaimus lolii* described in Australia was also transferred to *Ibipora* by Siviour and McLeod (1979). Fortuner and Luc (1987) proposed *Ibipora*, however, as a junior synonym of *Belonolaimus*, and *B. lineatus* and *B. lolii* were placed back in

Belonolaimus. They also transferred *I. anama* and *I. jara*, which were originally described by Monteiro and Lordello (1977), to *Belonolaimus*. On the other hand, *B. hastulatus* was transferred to *Tylenchorhynchus*. Nine species of *Belonolaimus* are reported at present: *B. gracilis*, *B. longicaudatus*, *B. euthychilus*, *B. maritimus, B. nortoni, B. anama*, *B. jara*, *B. lineatus*, and *B. lolii*.

Host Range

Many different economically important crops including vegetables, agronomic crops, grasses, and forest trees are hosts to *B. longicaudatus*. *Belonolaimus longicaudatus* was associated with cotton, corn, soybean, and peanut in the vicinity of Holland, Virginia (Owens,1951). Christie et al. (1952), established the pathogenicity of *B. longicaudatus* on strawberry, celery, and sweet corn, and pointed out the importance of this nematode in agriculture. Graham and Holdeman (1953) in South Carolina, investigated the occurrence of *B. longicaudatus* in seven counties, and reported that the nematode caused serious losses to cotton, corn, soybean, and cowpea, but not to tobacco.

Belonolaimus longicaudatus also is associated with citrus in Florida (Christie, 1959; DuCharme, 1954; Suit et al., 1953). *Belonolaimus euthychilus* (Rau, 1963) as well as *B. gracilis* and *B. longicaudatus*, were detected in citrus plantings in Florida (Esser and Simpson, 1984). *Belonolaimus gracilis* was collected from pine seedlings in Ocala, Florida (Steiner, 1949). *Belonolaimus longicaudatus* causes problems in turfgrasses in Florida (Perry and Smart, 1970; Rivera, 1963), and was recently shown to be pathogenic on cotton and potato (Crow et al., 2000), and has been found associated with many weeds (Esser, 1976; Holdeman, 1955; Kerr and Wysong, 1979).

Holdeman (1955) summarized the hosts of *B. longicaudatus* based on surveys from different geographical locations. He listed peanut, cotton, soybean, bean, corn, sweet potato, oat, onion, pepper, strawberry, pine tree, citrus, Austrian winter pea, winter pea, millet, celery, cowpea, lima bean, clover, lespedeza, and several grasses including Sudan, Bermuda, St. Augustine, and centipede grasses. However, watermelon and tobacco were non-hosts.

Robbins and Barker (1973) tested the host range among North Carolina and Georgia populations of *B. longicaudatus*. They categorized the populations into excellent hosts, good hosts, poor hosts, non-hosts, and differentiating hosts. The excellent hosts for *B. longicaudatus* were Chinese elm, Johnson grass, muscadine grape, pecan, strawberry, white clover, corn, hairy crabgrass, potato, pearl millet, and soybean. The good hosts were bentgrass, Japanese holly, oat, peach, peanut, wild carrot, and carrot. The poor host plants were camellia, cocklebur, cotton, gladiolus, Japanese holly, jimson weed, and lambsquarters, and the non-host plants were asparagus, buckhorn plantain, okra, pokeweed, sandbur, tobacco, and watermelon. There were some groups of plants that reacted differently to the North Carolina and Georgia populations of *B. longicaudatus*. These were bermudagrass, bush bean, curled dock, eggplant, fescue, highbush blueberry, lettuce, turnip, loblolly pine, onion, rabbit eye blueberry, wild garlic, and cabbage. Esser (1976) reported 116 hosts, and 27 resistant or non-host plants for *B. longicaudatus*.

Geographical Distribution

The distribution of *B. longicaudatus* was believed limited to the coastal plain region of the southeastern United States (Christie et al., 1952; Graham and Holdeman, 1953; Holdeman, 1955; Owens, 1951). According to Holdeman (1955), the presence of

B. longicaudatus was confirmed in Florida, Georgia, South Carolina, North Carolina, and Virginia. Possible existence was detected in Alabama, Mississippi, and Louisiana. No *B. longicaudatus* had been found, however, in Arkansas, Tennessee, or Kentucky until 1955.

Later, *Belonolaimus* species were reported from other locations within the United States: New Jersey (Hutchinson and Reed, 1956; Myers, 1979), Arkansas (Riggs, 1956), Kansas (Dickerson et al., 1972), Nebraska, Texas (Christie, 1959; Norton, 1959; Wheeler and Starr, 1987), and California (Mundo-Ocampo et al., 1994). Geographically, *Belonolaimus* spp. are known to most of the Atlantic and Gulf coast states, and more recently some areas in the mid-central United States. Nebraska marks the most northern location, whereas California marks the most western location. Except for the *Belonolaimus* sp. reported in Nebraska most sting nematodes in the United States were believed to be *B. longicaudatus*. The morphology of the *Belonolaimus* sp. isolate found in Nebraska indicated that they were morphological similar to that of *B. nortoni* (Kerr and Wysong, 1979).

Belonolaimus longicaudatus also has been reported from the Bahamas, Bermuda, Puerto Rico (Perry and Rhoades, 1982), and Costa Rica (Lopez, 1978). It is assumed that *B. longicaudatus* on golf course turfgrasses located in the Bahamas, Bermuda, and Puerto Rico were introduced on turfgrass sod from Florida and Georgia.

Intraspecific Variations

The pathogenicity of *B. longicaudatus* was tested on many crops such as peanut (Good, 1969; Owens, 1951; Perry and Norden, 1963), cotton (Graham and Holdeman, 1953; Crow et al., 2000), potato (Crow et al., 2000), strawberry, celery, sweet corn (Christie, 1952), collard, kale, cauliflower (Khuong, 1975), citrus (Suit and DuCharme,

1953), and grapefruit (Standifer and Perry, 1960). There are several reports about variations in pathogenicity, host range, and morphology among different geographical or host isolates (Abu-Gharbieh and Perry, 1970; Owens, 1951; Perry and Norden, 1963; Robbins and Barker, 1973; Robbins and Hirschmann, 1974). For instance, the Virginia isolates of *B. longicaudatus* were pathogenic to peanut (Owens, 1951), but Georgia and Florida isolates were not pathogenic to peanut (Good, 1968; Perry and Norden, 1963).

Variation in host range and morphology occurred among Florida isolates of *B. longicaudatus*. Abu-Ghabieh and Perry (1970) reported that three different isolates collected from different hosts in Florida showed various reactions to tested host plants. A citrus isolate reproduced well on rough lemon, whereas it failed to increase on strawberry. Two isolates from corn were able to reproduce on strawberry, but not on rough lemon. Morphological differences were not sufficient to warrant naming a new species of *B. longicaudatus*, but the variation in stylet length and tail length could aid in the differentiation of those populations. However, it must be noted that these populations fed on peanut and reproduced, in contrast to reports by Perry and Norden (1963) and Good (1968). On the other hand, morphometric variation of *B. longicaudatus* was reported among isolates taken from citrus orchards on Florida's central ridge, and populations of *B. longicaudatus* in northeastern Polk County tended to have longer stylets than tails, unlike all other populations (Duncan et al., 1996).

In 1973, Robbins and Hirschmann characterized the variation among North Carolina and Georgia isolates of *B. longicaudatus* for morphology, cytology, and hybridization. In morphology, the Georgia isolate was distinguished by the presence of sclerotized vaginal pieces lacking in North Carolina females, and in a blunter wide-tail

shape than those of North Carolina females. Morphometrically, the body length, stylet length and tail length of the Georgia isolates were greater than those of the North Carolina isolates. Although no differences in chromosome number were detected between Georgia and North Carolina isolates, they failed to generate F_2 offspring in interpopulation mating tests. Among three Florida isolates of *B. longicaudatus*, collected from different locations, no significant differences were found in morphology (Abu-Ghabieh and Perry, 1970). However, morphological variation of *B. longicaudatus* was detected of isolates taken from among or within citrus orchards on the Florida central ridge area. Populations of *B. longicaudatus* collected from citrus grown in northeastern Polk County tended to have stylets longer than tails, which was unlike all other populations (Duncan et al., 1996).

Robbins and Barker (1973) reported that isolates of *B. longicaudatus* reacted differently to some hosts. For example, one Georgia isolate was able to reproduce on cucumber, and dandelion, whereas three North Carolina isolates could not. Another important difference was that the reproductive rate of the Georgia isolate was significantly greater than that of the three North Carolina isolates. Also, they found that both the North Carolina and Georgia isolates reproduced on peanut, which is in contrast to previous reports from Perry and Norden (1963), and Good (1968).

In summary, there is wide intraspecific variation in the morphology, morphometry, and host range of putative *B. longicaudatus* isolates when they originate from different hosts or different geographic locations.

CHAPTER 3
BIOLOGICAL CHARACTERIZATION OF *BELONOLAIMUS LONGICAUDATUS*

Introduction

Belonolaimus longicaudatus (Rau, 1958) is an economically important plant parasite that attacks many varieties of agronomic and horticultural crops (Christie, 1953; Esser, 1976; Holdeman, 1955; Robbins and Barker, 1973). The significance of *B. longicaudatus* in agriculture was recognized following the Christie et al. (1952) report of ectoparasitism and pathogenicity of *B. longicaudatus* on strawberry, celery, and sweet corn.

Details on the biology, development, and behavior of *B. longicaudatus* have been poorly studied due to difficulties in rearing and observing the nematodes *in vitro*. Several studies on the biology of *B. longicaudatus*, conducted under greenhouse and field conditions, have resulted in reports on the life cycle and reproductive rate of Florida, Georgia, and North Carolina populations (Boyd and Perry, 1971; Perry, 1964; Robbins and Barker, 1974; Smart and Nguyen, 1991).

In Florida field studies, *B. longicaudatus* reproduced better at 29.4 °C than at 26.7 °C (Boyd and Perry, 1971; Perry, 1964). North Carolina and Georgia populations reproduced well in the range between 25 °C and 30 °C. The reproduction of the Georgia population was greatest at 30 °C, whereas reproduction of the North Carolina population was reduced at 30 °C (Robbins an Barker, 1974). In a greenhouse study, the life cycle *of B. longicaudatus* was completed in about 28 days (Smart and Nguyen, 1991). Recently,

in-vitro cultivation of *B. longicaudatus* made it possible to complete more detailed information on the life cycle and behavioral characteristics of a California isolate of *B. longicaudatus* (Huang and Becker, 1997; 1999). When cultured on excised corn roots, the life cycle was completed in a month at 26 °C to 27 °C (Huang and Becker, 1997) and 24 days at 28 °C. Mating occurred after the females finished the last molt and lasted 6 to 10 minutes (Huang and Becker, 1999). All juvenile stages as well as the adult stage of both genders fed on the root meristem (Huang and Becker, 1997).

The main objective of this study was to determine whether there are differences in developmental time among five isolates of *B. longicaudatus*. The detailed biological characteristics as well as development of each isolate was observed by in-vitro culture of *B. longicaudatus* on excised roots grown in Gamborg B-5 medium. In addition, a hybridization test was conducted to examine reproductive compatibility or incompatibility among five different isolates of *B. longicaudatus*.

Materials and Methods

Nematode Isolates

Five isolates of *B. longicaudatus* were collected from different geographical locations and different hosts. Three isolates came from different locations in Florida; HA isolate from potato at the Yelvinton farm, Research and Education Center, University of Florida, Hastings; GV isolate from bermudagrass at the Gainesville Golf and Country Club Gainesville; and LA isolate from citrus grown in block four, Citrus Research and Education Center, University of Florida, Lake Alfred. Others were GA isolate from cotton at the Rural Developmental Center, Tifton, Georgia, and NC isolate from corn

in Scotland County, North Carolina. *Dolichodorus miradvulvus* isolate was obtained from potato at the Yelvinton farm, Research and Education Center, University of Florida, Hastings, FL, and used as a control nematode for developmental comparisons among different isolates of *B. longicaudatus*.

Greenhouse Culture of Nematode

Each isolate was established on bermudagrass (*Cynodon dactylon* (L.) Pers) in the greenhouse. Bermudagrass was grown vegetatively in 25 cm-diameter clay pots filled with pasteurized sandy soil (95.5% sand, 2.0% silt, and 2.5% clay). Nematodes were extracted by the Baermann method (Ayoub, 1977), and females and males were handpicked. The inoculation level for each was 100 to 200 nematodes per pot. The pots were maintained in a greenhouse at 25 ± 5 °C and fertilized once a week with a 20-20-20 N-P-K soluble fertilizer.

Artificial Culture of Nematodes

After establishing all isolates of *B. longicaudatus* on bermudagrass, the nematodes were transferred to sweet corn (*Zea mays* L. cv. Silver Queen) cultured in Gamborg B-5 medium (Huettel and Rebois, 1985). Corn seeds were placed in sterilized disposable petri-dishes (100 × 15 mm) and surface sterilized with 95% ethanol for 3 minutes and 0.5% NaOCl for 10 minutes. The sterilized seeds were transferred onto 1.2% water agar and germinated at 28 °C in a Florida Reach-In chamber (Walker et al., 1993). Gamborg B-5 medium was prepared as a mixture of agar (1.3%) and Gamborg B-5 media (23%), which was adjusted to a pH of 5.2 to 5.6. The Gamborg B-5 medium-agar mixture was autoclaved for 15 minutes at 253 °C and 17 KPa. Two days after the

corn seed incubation, germinated corn roots were cut into 3 to 4 cm pieces with use of a sterile disposable knife and transferred onto the Gamborg B-5 medium. *Belonolaimus longicaudatus* were extracted from soil by the Baermann method, and females and males were handpicked. Approximately 100 nematodes were placed in a 1.5 ml micro-centrifuge tube filled with sterile distilled water, and centrifuged three times for 2 minutes at 10,000*g*. After centrifugation, the nematodes were transferred onto a 28-µm–pore- opening sieve (500-mesh) that had been autoclaved previously, and washed with 1 liter of sterile distilled water. Fifteen females and ten males were individually transferred onto each plate (60 × 15 mm). The plates containing nematodes were maintained at 28 °C in a Florida Reach-In chamber.

Comparisons of Developmental Patterns

Once females started to lay eggs, the developmental process (from egg to adult) was observed under an inverted microscope, and recorded daily. Specimens were photographed with an Olympus (OM-2N) camera. The observation of nematode development for each isolate was replicated 10 times and each stage-developmental time and total developmental time were subjected to statistical analysis and means were compared by Duncan's multiple-range test.

The time required in egg development (single-cell egg to hatch) was compared among each isolate at 18 °C, 23 °C, and 28 °C. Five eggs of each isolate were randomly selected in five different plates at each temperature, and developmental time for each was recorded.

Behavioral Characteristics of *B. longicaudatus*

Feeding, oviposition, and mating behavior of *B. longicaudatus* were observed on excised corn roots cultured in Gamborg-B-5 media with the use of an inverted microscope at magnifications of 20×, 100×, and 400×. The behavioral characteristics were recorded and photographed.

Hybridization Test

All isolates of *B. longicaudatus* were cultured on cotton (*Gossypium hirsutum* L. cv. Sure grow 125) for 2 months in a greenhouse and different developmental life stages were extracted by the Baermann method. For the hybridization test, 10 each of J4 females, J4 males, and adult males were picked from each isolate. Ten J4 females of the GA, LA, NC, HA, and GV isolates were combined with ten J4 males and 10 adult males from each of the same isolates to give a total of 25 combinations. The experimental design was a completely randomized block, and the test was repeated.

The host plant was 2-week-old cotton (*Gossypium hirsutum* L. cv. Sure grow 125). Seedlings were germinated under a moist paper towel for 3 days at room temperature and then transferred to Containers (Stuewe and Sons, Inc. Corvallis, OR) (1.5-diam. × 8.5 depth) filled with pasteurized sandy soil (95.5% sand, 2.0% silt, and 2.5% clay). The seedlings were placed at 28 °C in a Florida Reach-In chamber until inoculation. The nematodes were added to each by placing them in a 1 cm-wide × 5 cm-deep hole. After inoculation, the plants were returned to the Florida Reach-In chamber at 28 °C, and harvested 45 days later. Nematodes were extracted by the centrifugal flotation

method (Jenkins, 1964), and the final number of nematodes including juveniles was counted.

Results

Development and Behavior of *B. longicaudatus*

The development and behavior of *B. longicaudatus* were observed in *in-vitro* cultures of nematodes incubated at 28 °C. Females started to lay eggs 2 or 3 days after inoculation. The female reproductive system of *B. longicaudatus* is didelphic and amphidelphic. The eggs are squeezed from the uteri to the outside and deposited as single eggs (Fig. 3-1). Each female lays two eggs (Fig. 3-2, A), each produced separately from the paired ovaries at 30 second to 20 minute intervals. A single female was observed to lay nine to ten eggs over a 10 to 15 hour period (Fig. 3-2, A and B). The time required for single egg deposition from the uterus was approximately 80 to 100 seconds, but in some cases it took only 35 to 40 seconds. Deposited eggs were usually one-celled. After 2 to 3 hours, the one-celled stage divided to the two-celled stage, and 3 to 4 hours later, the two-celled stage divided to the four-celled stage. The gastrulation stage required approximately 24 to 36 hours and varied among the nematode isolates. After the gastrulation stage, the tadpole stage was observed. The J1 was observed 48 to 60 hours following egg deposition. The first molt occurred inside the egg shell, and took less than 24 hours. The total time of embryogenesis from the one-celled stage to J2 hatch ranged from 70 to 96 hours. Figure 3-3 shows the process of embryogenesis of *B. longicaudatus* from the one-celled stage to J2.

Most eggs were laid randomly throughout the culture media, however once the J2 hatched they moved quickly to the roots where they congregated around root hairs (Fig. 3-4, A). The J2 were the most active stage in their body movement, however, their feeding activity was less than other developmental stages and they were attracted to root hairs (Fig. 3-4, A) or root-cell debris (Fig. 3-4, B) rather than by root meristems (Fig. 3-4, C). It was not determined whether feeding was essential for the J2 to develop to the J3. After 3 to 5 days, most J2 began the second molt. The J2 were easily distinguished by their dark color, and fat-body shape. Their body movement slowed and stopped completely, which was the initial sign the second molt was beginning. During the molting process their bodies laid in a straight plane (Fig. 3-5, A), were fully curved (Fig. 3-5, B), or slightly curved (Fig. 3-5, C). The stylet cone was replaced with a new one, and the old stylet cone was shed (Fig. 3-6). The formation of the stylet shaft and knobs was never observed. The second molt took 2 days for completion for all isolates of *B. longicaudatus*.

The J3 moved immediately to root meristems of major roots or lateral roots. Their body movement was slow and remained so for the duration of the developmental period. Feeding was observed frequently lasting 6 hours and up to almost 10 hours. The J3 stage lasted a comparatively shorter time than other developmental stages. Two to 3 days passed before the third molt occurred. The distinctive molting sequence was identical to that for the second molt. The J3 became plumper in shape, dark in color, and their movement ceased. During the third molt, the stylet cone was shed with the old cuticle, and most J3 had a full-circle body shape. The third molt lasted 2 days.

The J4 stage lasted for 4 to 6 days until the initiation of the fourth molt. Feeding was very active during this developmental stage. Females and males were distinguished by the formation of a genital primordium. The final molt was similar in process to that of the second and third molts with the exception of the formation of the reproduction system (Figs. 3-7 and 3-8). The vulva and vagina of the female, and the bursa and spicules of the male were fully developed at the end of the fourth molt. This molting process took more than 2 days, which was longer than the second and third molts.

Mature males and females were observed feeding on cells along the root meristems (Fig. 3-9). All developmental stages of *B. longicaudatus* were more attracted to the root meristem in major (Fig. 3-9, A) and lateral roots (Fig. 3-9, B and C), however, a few nematodes were observed feeding along the cell-elongation region (Fig. 3-10, A). Cell necrosis and cessation of root growth followed nematode feeding, which resulted in roots with a "stubby" appearance. These symptoms were more pronounced after multiple feedings by many nematodes. Nine to ten nematodes (both adults and juveniles) were observed feeding along a root system (Fig. 3-10, B). During feeding, the stylet cone penetrated deeply into the root cells reaching the endodermis near the vascular system of young corn roots. Thus, *B. longicaudatus* not only feeds on the cortex, but also on the endodermis of corn roots. During feeding, the number of pulsations of the median bulb was estimated at 167 to 174 per minute.

Right after the fourth molt, there was attraction between opposite sexes. Distinctive mating behavior such as strong rubbing, touching, and twisting were observed between males and females (Fig. 3-11) with a duration lasting no more than 20 minutes.

Mating was observed right after the fourth molt and appeared to occur in a very limited time frame.

Variation of Developmental Times Among Isolates of *B. longicaudatus*

The NC isolate required 25 days for completing its development from egg to adult, which was the longest period for any isolate (Fig. 3-12, and Table 3-1) ($P \leq 0.05$). The second longest period was 22.9 days for the LA isolate ($P \leq 0.05$). Among the GA, GV, and HA isolates, there was no difference in developmental times with each lasting 18.1, 19.5, and 19.2 days, respectively.

The NC isolate had a longer egg developmental time than other isolates ($P \leq 0.05$). Both NC and LA isolates had longer J2 periods than those of the GA, GV, and HA isolates. The molting time of all isolates was very consistent and there was no difference among isolates, however the fourth molt took longer than the second and third molts. There was a greater range of variation in egg developmental time and the J4 period than J2 and J3 periods, and the J3 period showed the smallest variation among all developmental stages.

At 28 °C, the time for egg development was different among NC, LA, and GA isolates (Table 3-2) ($P \leq 0.05$). The eggs of the NC isolate took 4.6 days to develop to J2, which was the longest time among all *B. longicaudatus* isolates. The LA isolate showed the second longest egg developmental time with an average of 4 days, whereas the GA isolate had the shortest egg developmental time with 3.2 days. At 18 °C, the GA isolate required 8 days for development, which was shorter than any other isolate ($P \leq 0.05$). There was no difference in development among *B. longicaudatus* isolates at 23 °C,

and the mean egg developmental time ranged from 4.8 days (GA) to 5.6 days (LA). At 33 °C, most nematodes died within 7 to 10 days and no egg laying was observed.

Hybridization Test for Determining the Reproductive Compatibility or Incompatibility Among Different Isolates of *B. longicaudatus*

The existence of second-stage, or third-stage juveniles was used to indicate reproductive compatibility, whereas absence of juveniles was assumed to indicate incompatibility (Table 3-3). Generally all isolates of *B. longicaudatus* showed reproductive compatibility with each other except for GA (J4 female) and GV (J4 male), LA (J4 female) and HA (J4 male) in both experiments. The GV males showed comparatively poor ability to fertilize other females and generated a low number of offspring. In contrast, the NC males were attracted by all the other isolates of *B. longicaudatus* , and produced the highest number of juveniles.

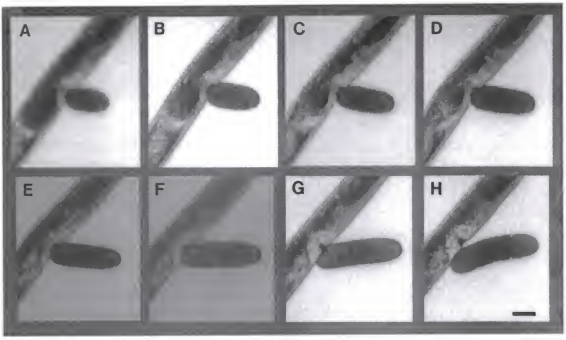


Figure 3-1. A series of photographs showing oviposition in a *Belonolaimus longicaudatus* female. A-G) The egg is squeezed from uterus to outside. H) A deposited single-celled egg. Scale bar is 10 µm.

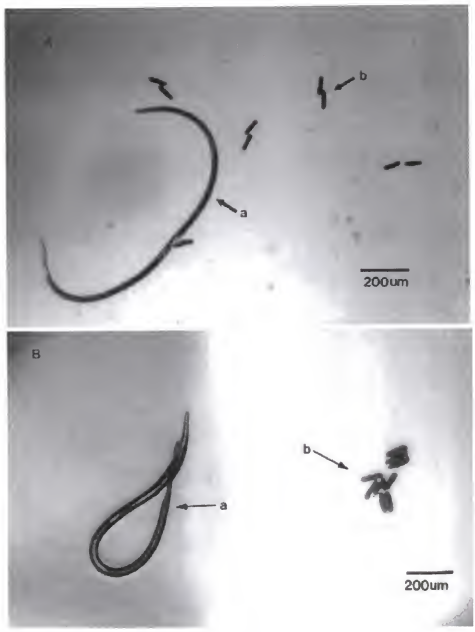


Figure 3-2. Pattern of egg deposition in a *Belonolaimus longicaudatus* female. A) Eggs deposited in pairs in sequence. B) A group of eggs deposited in sequence. a) Female. b) Eggs.

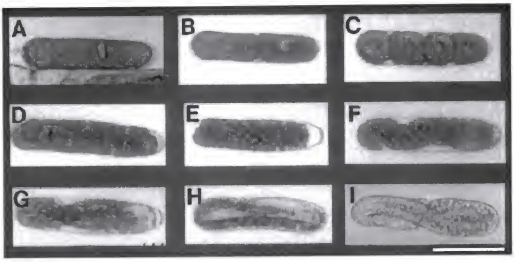


Figure 3-3. Egg embryogenesis of *Belonolaimus longicaudatus*. A) Single-cell stage. B) Two-cell stage. C) Four-cell stage. D) Multi-cell stage. E) Gastrulation stage. F) Tadpole stage. G) First-stage juvenile. H) First molt. I) Second-stage juvenile. Scale bar is 20 μm.

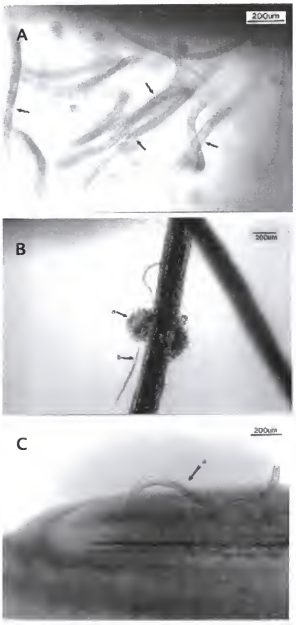


Figure 3-4. Behavioral characteristics of second-stage juveniles of *Belonolaimus longicaudatus*. A) Congregated second-stage juveniles around root hairs. B) Second-stage juveniles attracted by root-cell debris. a) Root-cell debris. b) Nematode. C) Second-stage juvenile feeding at root meristem. a) Nematode. Arrows in A indicate second-stage juveniles.

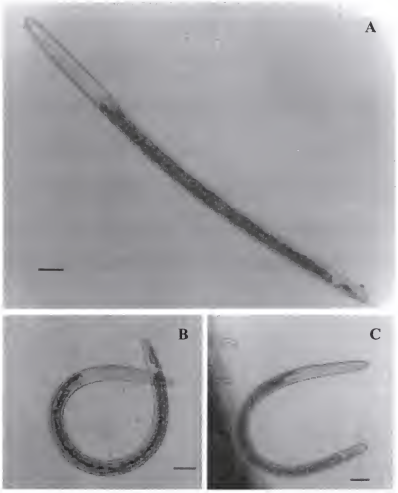


Figure 3-5. Molting process of a second-stage juvenile. A) Straight form. B) Full circle. C) C-shaped. Scale bar is 30 μm.

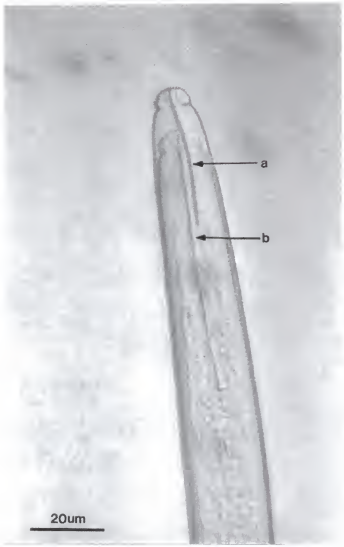


Figure 3-6. Molting characteristics of *Belonolaimus longicaudatus*. Old stylet cone is shed together with the old cuticle, and new stylet cone is replaced. a) Old stylet cone. b) New stylet cone.

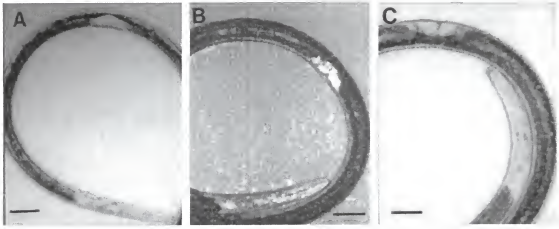


Figure 3-7. Formation of female reproductive organs during fourth molt. A) Early stage of fourth molt. B) Vagina becomes visible in the middle of the fourth molt. C) Fully developed vulva and vagina at the end of fourth molt. Scale bar is 40 μm.

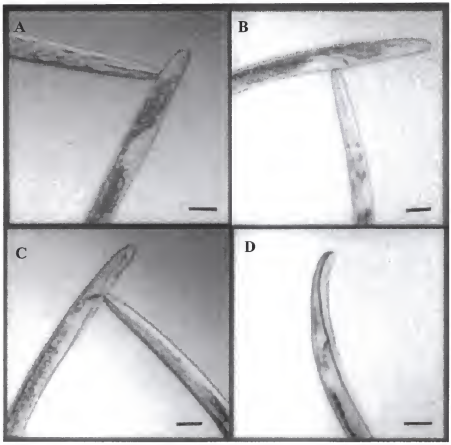


Figure 3-8. Formations of male reproductive organs during fourth molt.
A) Early stage of fourth molt. B) Middle of the fourth molt. Spicule is visible. C) End of fourth molt. Spicule and bursa are fully developed. D) Mature male. Scale bar is 40 μm.

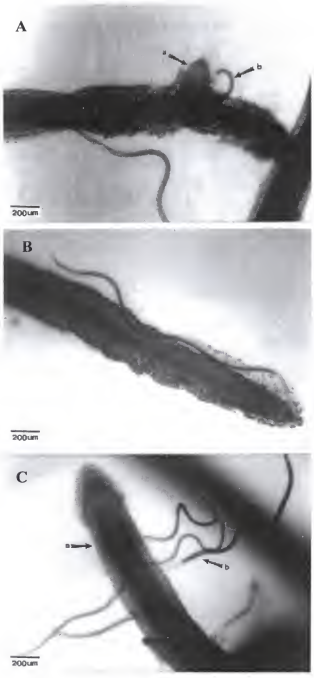


Figure 3-9. Feeding locations of *Belonolaimus longicaudatus* on corn roots. A) Major root meristem. B) Young lateral root. a) Root meristem, b) Nematode. C) Lateral root. a) Corn lateral root. b) Nematode.

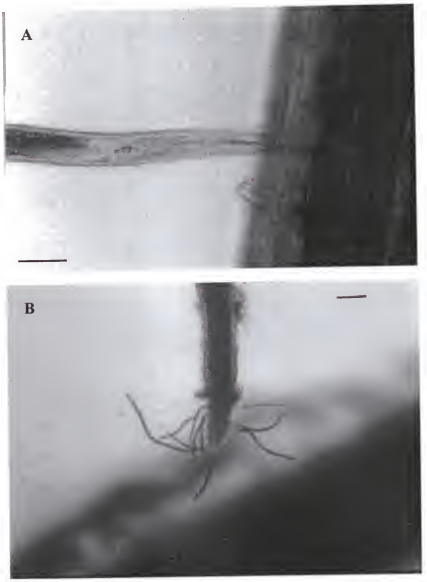


Figure 3-10. Characteristics of feeding behavior in *Belonolaimus longicaudatus*. A) Feeding occurring in higher location of root growth region. B) Multiple feeding by nine nematodes on one root meristem. Scale bar on A and B is 50 µm and 30 µm, respectively.

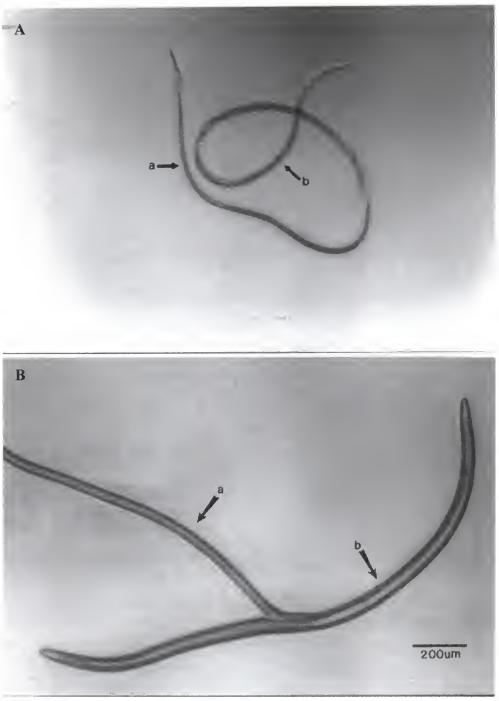


Figure 3-11. Mating behavior of *Belonolaimus longicaudatus* at the end of the fourth molt. A) Attraction between male and female. B) Copulation. a) Male. b) Female.

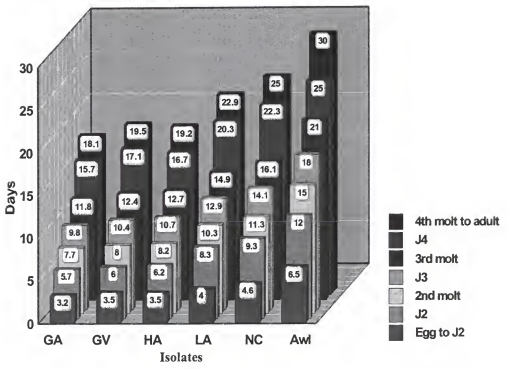


Figure 3-12. Comparative developmental patterns of five isolates of *Belonolaimus longicaudatus* and *Dolichodorus miradvulvus* (awl nematode). Each bar shows the different developmental stages of nematode, and molts. The numerical value on top of each bar indicates the time required from egg to adult. The isolates of *B. longicaudatus* were collected from potato in Hastings, FL (HA), bermudagrass in Gainesville, FL (GV), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), corn in Scotland County, NC (NC), and *D. miradvulvus* was from potato in Hastings, FL.

Table 3-1. Comparisons of the developmental times among five different isolates of *Belonolaimus longicaudatus* cultured on excised corn roots grown in Gamborg B-5 medium at 28 °C (Units = day).

Developmental stages	Isolates					
	GV	HA	LA	GA	NC	CA[b]
Egg to J2	3.5 ± 0.53 [a] bc	3.5 ± 0.53 bc	4.0 ± 0.67 b	3.2 ± 0.42 c	4.6 ± 0.52 a	5
J2	2.5 ± 0.53 b	2.7 ± 0.48 b	4.3 ± 0.67 a	2.5 ± 0.53 b	4.7 ± 0.48 a	2
2nd molt	2.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00	2
J3	2.4 ± 0.52 ab	2.5 ± 0.53 ab	2.6 ± 0.52 a	2.1 ± 0.32 b	2.8 ± 0.42 a	3
3rd molt	2.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00	2.0 ± 0.00	2
J4	4.7 ± 1.25 bc	4.0 ± 1.15 c	5.4 ± 0.97 ab	3.9 ± 1.20 c	6.2 ± 1.03 a	4 - 5
4th molt to adult	2.4 ± 0.52	2.5 ± 0.53	2.6 ± 0.52	2.4 ± 0.52	2.7 ± 0.48	2 - 3
Total time	19.5 ± 1.43 c	19.2 ± 1.75 c	22.9 ± 1.52 b	18.1 ± 2.02 c	25.0 ± 1.89 a	20 - 22

The isolates of *Belonolaimus longicaudatus* were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), and corn in Scotland County, NC (NC). The CA isolate of *B. longicaudatus* was collected from turfgrass in Rancho Mirage, CA (Xiang and Becker, 1999).

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$). Lack of letter denotes non-significance.

[a] Mean number of days and the standard deviation for each developmental stage.

[b] The developmental time for the CA (California) isolate was described by Xiang and Becker (1999).

Table 3-2. Comparisons of the egg developmental time for five different isolates of *Belonolaimus longicaudatus* cultured on excised corn roots grown in Gamborg B-5 medium at different temperatures (Units = days).

	Isolates				
Temperature	GV	HA	LA	GA	NC
18 °C	10.0 ± 0.71[a] a	9.2 ± 0.45 a	9.4 ± 0.55 a	8.0 ± 0.71 b	9.6 ± 0.55 a
23 °C	5.2 ± 0.45	5.0 ± 0.71	5.6 ± 0.55	4.8 ± 0.84	5.4 ± 0.55
28 °C	3.5 ± 0.53 bc	3.5 ± 0.53 bc	4.0 ± 0.67 b	3.2 ± 0.42 c	4.6 ± 0.52 a
33 °C	-[b]	-	-	-	-

The isolates of *Belonolaimus longicaudatus* were collected from cotton in Tifton, GA (GA), potato in Hastings, FL (HA), bermudagrass in Gainesville, FL (GV), citrus in Lake Alfred, FL (LA), and corn in Scotland County, NC (NC).

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$). Lack of letter denotes non-significance.

[a] Mean number of days and standard deviation for each developmental stage.

[b] No eggs were observed.

Table 3-3. Determination of compatibility or incompatibility among different isolates of *Belonolaimus longicaudatus* based on hybridization test after 45 days.

Male[b] isolates	Replication	Female isolates[a]				
		GV	HA	LA	GA	NC
GA	1	m[d]	+	+	+[c]	m
	2	++	+	++	+	+
HA	1	++	+	-	++	++
	2	+	++	-	+	-
GV	1	+	+	+	-	-
	2	+	+	-	-	+
LA	1	-	+	+	+	+
	2	+	++	+	+	+
NC	1	+++	+	+++	+++	+
	2	+	+	+	m	+

The isolates of *Belonolaimus longicaudatus* were collected from cotton in Tifton, GA (GA), potato in Hastings, FL (HA), bermudagrass in Gainesville, FL (GV), citrus in Lake Alfred, FL (LA), and corn in Scotland County, NC (NC).

[a] J4 females (n = 10)

[b] Combination of male (n = 10) and male forth-stage juvenile (n = 10) of each isolate.

[c] + The number of juveniles are < 5.
++ The number of juveniles are between 5 to 10.
+++ The number of juveniles are > 10.
- No offspring (juveniles).

[d] Missing data.

Discussion

The developmental time (egg to adult) of *B. longicaudatus* on sweet corn varied among five isolates collected from different hosts and regions of the southeastern United States, and ranged from 18.1 days for the GA isolate to 25 days in the NC isolate at 28 °C. Robbins and Barker (1973) reported differences in the reproductive rate between Georgia and North Carolina isolates. According to their reports, three North Carolina isolates had a lower rate of reproduction than a Georgia isolate when tested on several different host plants. In addition, the reproductive rate of North Carolina isolates was slightly reduced at 30 °C, whereas the Georgia isolate increased in population numbers at this temperature (Robbins and Barker, 1974). Thus, geographically separated isolates have different biological characteristics in reproductive rates and in temperature preferences.

Recently, Huang and Becker (1999) reported the life cycle of a *B. longicaudatus* isolate collected from turfgrass in Rancho Mirage, California and they found that this isolate of *B. longicaudatus* completed its life cycle in 24 days at 28 °C in gnotobiotic culture. The developmental time of the California (CA) isolate of *B. longicaudatus* ranged from 20 to 22 days, which was similar to that for the GV, HA, and LA isolates. Thus, biological characteristics of the CA isolate seemed closer to the FL isolates than the GA or NC isolates. Cherry et al. (1997) hypothesized that California *B. longicaudatus* was introduced from Florida based on PCR-RFLP analysis of ITS-1. The California isolate was distinguished by a longer egg developmental time (5 days) than occurred for FL and GA isolates, and a shorter J2 period (2 days) than all the other

isolates. However, there were no differences in molting time among the CA, FL, GA, and NC isolates.

The total developmental time of *B. longicaudatus* ranged from 18 to 25 days at 28 °C, which was comparatively shorter than for other ectoparasitic nematodes. *Hoplolaimus indicus* completed their development in 24 to 33 days under i*n-vitro* condition at 28 °C to 32 °C (Dasgupta et al., 1970). The developmental time of *Criconemoides xenoplax* was 23 to 31 days under laboratory conditions (Seshadri, 1964).

There is no information on egg embryogenesis of *B. longicaudatus*. In these experiments, development of a single-celled egg was observed through two-celled, four-celled, multi-celled, gastrulation and tadpole stages, first-stage juvenile, and first molt. The gastrulation stage was not only the longest period among the embryonic stages but also the most varied. Approximately 4 to 5 days were required for the egg development of *B. longicaudatus*. *Helicotylenchus multicinctus* took 4 to 6 days for egg development, which was close to that for *B. longicaudatus* (Orion and Bar-Eyal, 1995), whereas the embryogenesis of *H. indicus* lasted 8 to 9 days (Dasgupta et al., 1970) and *C. xenoplax* lasted 11 to 13 days (Seshadri, 1964).

There are similarities in some developmental characteristics between *H. indicus* and *B. longicaudatus*. For instance, feeding was essential in both species for development of juveniles and necessary for egg laying of the females. Emerged J2 of both species were found to congregate in the young roots hair (Dasgupta et al., 1970). However, the time intervals between laying the first and second eggs was shorter in *B. longicaudatus* (30 seconds to 20 minutes) than for *H. indicus* (15 minutes to 3 hours), and the feeding time was longer in *H. indicus* (more than 72 hours) than in *B. longicaudatus*

(6 to 10 hours). For *H. indicus*, sometimes cell division in the eggs started within the uterus and often J2 hatched within the body of the female. This was never observed in *B. longicaudatus*. Another ectoparasitic nematode, *C. xenoplax*, is different from *B. longicaudatus* in that cleavage of eggs in the uterus was common and feeding was unnecessary for the laying of eggs (Seshadri, 1964).

Hybridization tests documented reproductive compatibility among different isolates of *B. longicaudatus* except for the GA (female) x GV (male) and the LA (female) x HA (male). However, interpopulation matings of the GV (female) x GA (male) and the HA (female) x LA (male) resulted in F_1 offspring. Therefore, all five isolates of *B. longicaudatus* showed reproductive compatibility with each other. Robbins and Hirshmann (1974) reported on interbreeding tests between North Carolina and Georgia isolates. They found the number of F_1 progeny ranged from 0.7 to 32.3 individuals between these isolates. The number of progeny was higher in North Carolina (female) x Georgia (male) than Georgia (female) x North Carolina (male). Even though North Carolina and Georgia isolates produced greater numbers of F_1 progeny, they failed to produce F_2 progeny (Robbins and Hirshmann, 1974). Hybridization studies reported in this manuscript were terminated before production of F_2 progeny and conclusions cannot be made regarding fertility of the F_1 produced.

CHAPTER 4
MORPHOLOGICAL CHARACTERIZATION OF *BELONOLAIMUS LONGICAUDATUS*

Introduction

Morphological variation of *B. longicaudatus* has been reported with the characterization of average stylet length, shape of stylet knobs, tail shape, number of tail annules, stylet length-tail length ratio, degree of head constrictions, and presence or absence of sclerotized pieces in the vagina (Abu-Ghabieh and Perry, 1970; Duncan et al., 1996; Rau and Fassuliotis, 1970; Robbins and Hirshmann, 1974).

Robbins and Hirshmann (1974) reported on morphology-morphometrics of each of three isolates from North Carolina and Georgia. The cephalic region of the Georgia isolates was more massive than that of North Carolina isolates and opposed sclerotized vaginal pieces were observed only in the Georgia isolates. In addition, tails of females from Georgia were typically wider, less tapered and more bluntly rounded than those of females from North Carolina. The stylet knobs of nematodes from North Carolina were typically tear-dropped or kidney shaped, whereas those of the Georgia isolates were typically rounded or oval. All Georgia isolates (females) of *B. longicaudatus* were significantly different from all North Carolina (female) isolates for "a" and "c" ratios, stylet and stylet cone lengths, and excretory pore to head. However, there were no differences in total body length, tail length and stylet length-tail length ratio among the isolates. On the other hand, morphological variation of *B. longicaudatus* was detected

among isolates taken from citrus orchards on the Florida central ridge area (Duncan et al., 1996). Populations of *B. longicaudatus* collected from citrus grown in northeastern Polk County tended to have stylets longer than tails, which was unlike all other populations. Among three Florida isolates of *B. longicaudatus* collected from different locations and hosts, no significant differences were found in morphology (Abu-Ghabieh and Perry, 1970).

Based on these previous studies it appears that there are variations in morphology among isolates identified as *B. longicaudatus*. However, none of these studies present morphological comparisons between *B. longicaudatus* isolates collected from Florida, Georgia, and North Carolina. In this study, the morphology of isolates collected from Florida, Georgia, and North Carolina were compared. The morphological data was compared with that of the original published data.

Materials and Methods

The *B. longicaudatus* isolates and the procedures for their cultivation on excised corn roots were the same as described in chapter 3.

Specimen Preparation

The females (right after 4th molt) and second-stage juveniles (J2) were hand picked, and prepared for measurements. The J2 were measured after being killed by gentle-heat treatment, while females were measured following several steps of fixation. For fixation, the females were placed in hot (60 °C) TAF (triethanolamineformaldehyde) and left at room temperature for 24 hours. The TAF was replaced with ethanol-glycerin

(ethanol 20%:glycerin 1%:water 79%) for 12 hours and ethanol-glycerin (ethanol 95%:glycerin 5%) for 5 to 7 days.

Measurements of *B. longicaudatus*

Thirty-five females and thirty J2 from each isolate were measured by using a microscope fitted with a drawing tube. The body length and distance from vulva to anterior body end as a percentage of total body length (V%) of females were measured at 20× magnification, and other morphological characters were measured at 400× magnification. The J2 body length was measured at 100× magnification, and other morphological characters were measured at 400× magnification.

The following measurements, ratios, and characters were recorded for females: body length, greatest body width, stylet length, stylet cone length, stylet shaft and knobs length, distance from excretory pore to anterior body end, the length of esophagus, tail length, the height of head constriction, a (total body length-body width), b (total body length-length of esophagus), and c (total body length-tail length) ratios, stylet-tail ratio, and body length-stylet ratio. The ratio a, b, and c were calculated using de Man indices. Measurements for J2 included body length, greatest body width, stylet length, stylet cone length, stylet shaft and knobs length, and the height of head constriction. The morphological data from both female and second-stage juveniles were subjected to statistical analysis. In addition, all morphological data were directly compared to the original description of *B. longicaudatus*. The mean, standard deviation, and range were calculated, and the significant differences of those values among different isolates were compared by Duncan's multiple-range test.

Published Morphological Data

Morphological information presented in Tables 4-4 and 4-5 is presented to aid in comparison with the new data generated herein.

Results

Comparisons of Female Morphometry

Females of the LA and NC isolates were characterized by larger body length, greater body width, head to excretory pore distance, and tail length than those of other isolates (Table 4-1). The LA isolate was distinctive in having a longest stylet and a higher head constriction than all other isolates (Table 4-1) ($P \leq 0.05$). On the other hand, the GV isolate was smaller in body length, excretory pore, and stylet length (stylet cone, shaft, and knobs) (Table 4-1).

The GA and NC isolates' stylet length was intermediate among all isolates; however, the GA isolate had a larger stylet cone and shorter stylet shaft and knobs than the NC isolate. In tail length, the LA and NC isolates were separated from all other isolates but, based on c, the NC and HA isolates were differentiated from all other isolates with shorter tail proportion from total body length. In stylet-tail ratio, the GA, LA, and GV isolates showed a longer stylet than tail ratio, whereas the NC and HA isolates had a longer tail than stylet. The HA isolate was a thinner nematode than any of the other isolates by having the smallest body width and the largest a, and it had the most anteriorly located vulva position. Based on the b ratio, the NC and HA isolates have a comparatively longer esophagus than other isolates of *B. longicaudatus*.

Among the morphological characters, the largest variation was for the stylet cone lengths, stylet shaft and knob lengths, esophagus lengths, and a ratios, whereas the vulva position (%) had the least variation among isolates.

Comparisons of J2 Morphometry

The J2 from the five isolates showed different characteristics in body length, body width, stylet length, and head height (Table 4-2). The LA isolate was larger in stylet length and head height, whereas the GV isolate had a shorter stylet length compared with the other isolates ($P \leq 0.05$). In body length and body width, the NC isolate had the longest body and the widest body width, however, the GV isolate had the shortest body and smallest body width among all isolates ($P \leq 0.05$). The HA isolate was distinctive with a lower head constriction (Table 4-2) ($P \leq 0.05$).

The morphological variation of J2 was much larger than that for females, especially in head height and stylet length. There were no significant differences in the head heights of females among isolates except the LA isolate. However, the J2 showed variation in the ranges of head height and the HA isolate had the lowest head constriction among the isolates ($P \leq 0.05$).

Comparison of Female Stylet Knobs

Two different types of stylet knobs were observed among the five isolates of *B. longicaudatus*. The stylet knobs of the NC and LA isolates were typically teardrop or kidney-shaped, whereas the GA isolate was typically oval (Fig. 4-1). The HA and GV isolates were variable in that specimens from each had both round and kidney-shaped stylet knobs.

Comparison of Female Reproductive Organ

Opposed vaginal pieces were observed in all isolates of *B. longicaudatus* except for the GA isolate (Fig. 4-2). There were no vaginal pieces in the GA isolate, whereas morphological variation of the vaginal pieces occurred in other isolates. The vaginal pieces of the LA isolate were the most prominent and clearly recognized among all isolates. The HA and GV isolates possessed smaller vaginal pieces than the others, and that of NC isolate was weakly developed and not clearly recognized.

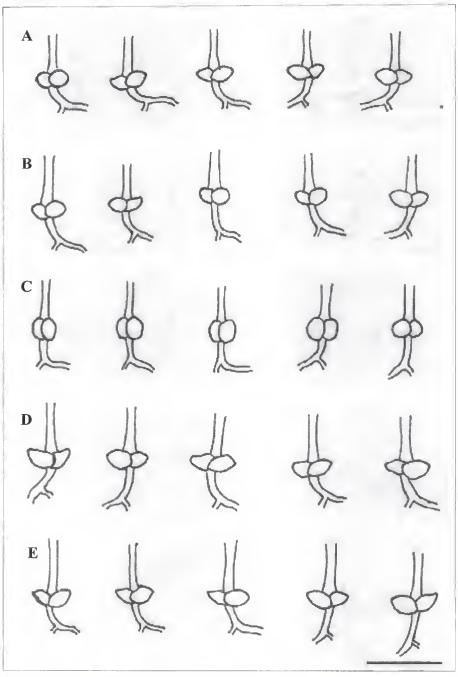


Figure 4-1. Morphological variation of the stylet knobs among five isolates of *Belonolaimus longicaudatus*. A) Gainesville isolate, B) Hastings isolate, C) Georgia isolate, D) Lake Alfred isolate, E) North Carolina isolate. Scale bar indicates 10 µm.

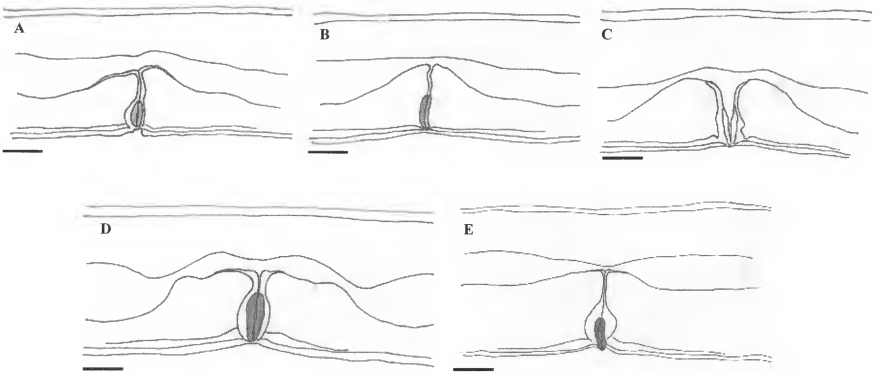


Figure 4-2. Morphological variation of female vaginal pieces among five isolates of *Belonolaimus longicaudatus*. A) Gainesville isolate, B) Hastings isolates, C) Georgia isolate, D) Lake Alfred isolate, E) North Carolina isolate. Scale bar indicates 10 µm.

Table 4-1. Measurements of females (n = 35) of five *Belonolaimus longicaudatus* isolates[a] taken from excised corn roots cultured on Gamborg B-5 medium at 28 °C.

	GV	HA	LA	GA	NC
	Mean, standard deviation, and range				
			Linear (µm)		
Body length	2,047.2 ± 148.2 c (1,757.6 - 2,424.2)	2,189.8 ± 178.6 b (1,878.8 - 2,606.1)	2,277.9 ± 163.4 a (1,909.1 - 2,681.8)	2,135.5 ± 88.3 b (2,000.0 - 2,348.5)	2,320.8 ± 133.6 a (2,030.3 - 2,515.2)
Body width	40.9 ± 3.5 b (36.4 - 51.5)	39.0 ± 2.5 c (34.9 - 45.5)	42.8 ± 3.0 a (34.9 - 48.5)	40.8 ± 2.2 b (36.4 - 45.5)	42.5 ± 4.3 a (36.4 - 53.0)
Stylet length	115.5 ± 7.0 c (103.0 - 132.6)	118.1 ± 6.4 c (107.6 - 129.6)	129.4 ± 9.4 a (114.4 - 171.2)	124.5 ± 7.6 b (106.1 - 145.5)	123.5 ± 6.7 b (109.1 - 139.4)
Stylet cone length	84.6 ± 5.8 d (72.7 - 98.5)	85.7 ± 5.3 cd (75.8 - 95.5)	94.9 ± 7.5 a (83.3 - 128.8)	91.4 ± 5.6 b (75.8 - 104.6)	88.2 ± 5.9 c (75.8 - 103.1)
Stylet shaft and knobs length	31.0 ± 2.1d (27.3 - 34.9)	32.5 ± 2.7c (25.8 - 37.9)	34.5 ± 3.1 ab (28.8 - 42.4)	33.1 ± 4.5 bc (27.3 - 54.6)	35.3 ± 1.8 a (31.1 - 39.4)
Excretory pore to head end	201.1 ± 16.1 c (165.2 - 247.0)	208.7 ± 20.0 bc (174.2 - 243.2)	226.9 ± 16.5 a (180.3 - 257.6)	209.2 ± 13.3 bc (180.3 - 228.8)	228.5 ± 19.5 a (154.6 - 257.6)
Tail length	115.0 ± 17.7 b (75.8 - 150.0)	134.7 ± 13.2 b (106.1 - 168.2)	127.4 ± 12.6 a (99.2 - 159.1)	124.0 ± 14.8 b (101.5 - 156.1)	142.1 ± 12.9 a (119.7 - 165.2)
Esophagus length	264.1 ± 18.9 d (228.8 - 309.1)	270.0 ± 20.5 b (222.7 - 310.6)	286.3 ± 17.6 c (233.3 - 321.2)	270.4 ± 16.1 c (237.9 - 297.0)	283.8 ± 20.1 a (221.2 - 319.7)

Table 4-1. – continued.

	GV	HA	LA	GA	NC
	Mean, standard deviation, and range				
Head height	10.9 ± 1.19 b (9.1 - 15.9)	10.7 ± 0.9 b (9.1 - 2.9)	11.8 ± 0.9 a (9.9 - 13.6)	10.9 ± 0.7 b (9.9 - 13.6)	10.6 ± 0.8 b (9.1 - 13.6)
a	50.3 ± 4.7 d (40.0 - 59.3)	56.3 ± 5.3 a (47.3 - 67.4)	Ratio[b] 53.4 ± 4.5 bc (42.0 - 65.2)	52.5 ± 3.2 cd (45.4 - 59.2)	55.2 ± 6.3 ab (41.7 - 69.2)
b	7.8 ± 0.6 b (6.5 - 9.1)	8.1 ± 0.6 a (7.1 - 9.2)	8.0 ± 0.8 ab (6.8 - 11.4)	7.9 ± 0.5 ab (7.0 - 9.1)	8.2 ± 0.5 a (7.2 - 9.9)
c	18.1 ± 2.6 a (14.0 - 25.2)	16.3 ± 1.2 b (14.2 - 18.3)	18.0 ± 1.4 a (14.6 - 20.6)	17.4 ± 1.9 a (13.7 - 20.9)	16.4 ± 1.5 b (13.9 - 19.5)
Stylet/tail	1.01 ± 0.2 a (0.8 - 1.5)	0.88 ± 0.1 b (0.7 - 1.1)	1.03 ± 0.1 a (0.8 - 1.5)	1.01 ± 0.1 a (0.8 - 1.2)	0.88 ± 0.1 b (0.7 - 1.1)
Body length/stylet	17.6 ± 0.2 b (15.7 - 20.6)	18.5 ± 1.2 a (16.6 - 21.1)	17.7 ± 1.4 b (14.9 - 19.8)	17.2 ± 1.0 b (13.8 - 21.9)	18.8 ± 1.3 a (16.1 - 21.9)
V	49.6 ± 2.2 ab (44.7 - 54.3)	49.2 ± 1.9 b (45.2 - 54.4)	Proportion[c] 50.6 ± 1.7 a (44.7 - 54.0)	50.6 ± 2.5 a (44.9 - 59.7)	50.5 ± 1.8 a (46.0 - 54.6)

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

[a] The isolates of *Belonolaimus longicaudatus* were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), and corn in Scotland County, NC (NC).

[b] a = Total body length per body width, b = Total body length per length of esophagus, and c = Total body length per tail length.

[c] Distance from vulva to anterior body end as a percentage of total body length.

Table 4-2. Measurements of second-stage juveniles (n = 30) of five *Belonolaimus longicaudatus* isolates[a] taken from excised corn roots cultured in Gamborg B-5 medium at 28 °C.

	GV	HA	LA	GA	NC
	Mean, standard deviation, and range (μm)				
Body length	535 ± 69 ab (410 - 693)	492.± 35 c (422 - 545)	515 ± 37 bc (449 - 615)	535 ± 30 ab (476 - 590)	543 ± 42 a (452 - 633)
Body width	24.8 ± 3.4 b (16.7 - 30.3)	23.1 ± 2.3 c (19.7 - 28.8)	22.9 ± 0.9 c (21.2 - 25.0)	23.2 ± 1.7 c (20.5 - 28.0)	27.8 ± 2.5 a (21.2 - 30.3)
Stylet length	63.1 ± 4.8 d (50.0 - 68.9)	65.7 ± 2.5 c (62.1 - 71.2)	70.7 ± 1.6 a (68.2 - 74.2)	67.8 ± 2.4 b (62.9 - 72.0)	65.2 ± 2.9 c (56.8 - 69.7)
Stylet cone length	44.5 ± 3.8 d (34.9 - 49.2)	46.5 ± 2.5 c (42.4 - 51.5)	50.7 ± 1.6 a (48.5 - 54.6)	48.5 ± 2.1 b (43.9 - 52.3)	45.8 ± 2.3 cd (39.4 - 50.0)
Stylet shaft and knobs length	18.5 ± 1.5 c (14.4 - 20.5)	19.1 ± 1.0 b (16.7 - 21.2)	20.0 ± 0.8 a (18.2 - 21.2)	19.3 ± 0.9 b (18.2 - 21.2)	19.4 ± 1.3 b (17.4 - 22.7)
Head height	7.3 ± 1.2 ab (6.1 - 8.3)	6.8 ± 0.9 d (6.1 - 7.6)	7.5 ± 0.9 a (6.8 - 8.3)	7.0 ± 0.7 ab (6.1 - 7.6)	7.2 ± 0.8 bc (6.1 - 9.1)

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$). Lack of letter denotes non-significance.

[a] The isolates of *Belonolaimus longicaudatus* were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), corn in Scotland County, NC (NC).

Table 4-3. Female morphological data of five species of *Belonolaimus* taken from the original descriptions (Unit = µm).

Species (Publication)	Body length	Stylet length	Tail length	a	b	c	V	N[b]
B. gracilis (Steiner, 1942)	2,015	157	120	52	6.1	19.2	52	1
B. gracilis (Rau, 1961)	1,900 (1,410-2,300)	152 (130-168)	78 (53-134)	49 (39-63)	6.7 (5.1-9.8)	23 (16-28)	53 (50-57)	61
B. gracilis (Rau, 1963)	2,014 (1,410-2,460)	153 (99-175)	89 (60-122)	49 (39-63)	6.7 (5.1-9.8)	23 (16-28)	55 (50-57)	209 (54)[c]
B. longicaudatus (Rau, 1958)	2,200 (2,000-2,600)	118 (100-133)	- [a]	65.4 (55.7-74.9)	8.4 (7.3-9.9)	16.1 (14.5-18.0)	50 (46-54)	22
B. longicaudatus (Rau, 1961)	2,500 (2,000-3,000)	127 (115-140)	156 (115-183)	64 (55.7-74.9)	9.04 (7.0-9.9)	18 (13-21)	49 (47-51)	31
B. longicaudatus (Rau, 1963)	2,509 (1,986-3,012)	127 (115-140)	154 (115-189)	64 (55-74)	9.0 (7.2-12.6)	18 (13-21)	49 (46-54)	136 (27)

Table 4-3. - continued.

Species (Publication)	Body length	Stylet length	Tail length	a	b	c	V	N[b]
B. euthychilus (Rau, 1963)	1,850 (1,426-2,094)	154 (131-168)	88 (56-148)	45 (39-62)	5.7 (5.2-6.1)	20 (15-17)	53 (50-57)	192 (62)
B. maritimus (Rau, 1963)	2,489 (2,052-2,994)	149 (119-171)	120 (83-155)	54 (43-67)	7.7 (6.6-8.7)	21 (17-24)	52 (47-56)	290 (96)
B. nortoni (Rau, 1963)	1,854 (1,604-2,124)	90 (78-98)	108 (84-130)	58 (51-64)	8.0 (6.9-9.3)	17 (15-18)	50 (49-53)	53 (17)

V Distance from vulva to anterior body end as a percentage of total body length.

a = Total body length per body width, b = total body length per length of esophagus, and c = Total body length per tail length.

[a] Missing data.

[b] Number of nematodes tested.

[c] Number of nematodes tested for a, b, and c.

Table 4-4. Published female morphological data of *Belonolaimus longicaudatus* isolates from Florida, Georgia, and North Carolina (Unit = µm).

Locality	Host plants	Body length	Stylet length	Tail length	Stylet/ Tail	a	b	c	Reference
Sanford, FL (27)[a]	Corn	2,509	127	154	64	9.0	18	16.1	(Rau, 1963)
Gainesville, FL (17)	Corn	2,097	124	119	-	52	7.5	17.2	(A & P, 1970)[d]
Fuller's Crossing, FL (19)	Citrus	2,274	118	131	-	64	7.9	17.6	(A & P, 1970)
Sanford, FL (16)	Corn	2,206	113	141	-	59	7.9	15.0	(A & P, 1970)
Polk, Co. FL (15)	Swingle citrumelo	2,545 (52)[b]	142(2)	131(4)	1.10(0.04)	-	-	-	(Duncan et al., 1996)
Polk, Co. FL (20)	Carrizo citrange	2,483 (43)	133(1)	132(3)	1.02(0.02)				(Duncan et al., 1996)
Manatee, Co. FL (16)	Sour orange-Rough lemon	2,589 (54)	128(1)	147(3)	0.88(0.02)	-	-	-	(Duncan et al., 1996)
Hillsborough, Co. FL (10)	Grass	2,557 (49)	121(2)	156(4)	0.79(0.03)	-	-	-	(Duncan et al., 1996)

Table 4-4. - continued.

Locality	Host plants	Body length	Stylet length	Tail length	Stylet/ tail	a	b	c	Reference
Tarboro, NC (24)	Cotton	2,096	107.3	111.9	0.97	66.0	12.6	18.8	(R & H, 1974)[e]
Dudley, NC (24)	Corn	2,161	110.0	115.8	0.95	64.4	11.9	18.7	(R & H, 1974)
Severn, NC (24)	Peanut	2,334	114.3	127.8	0.90	68.6	12.7	18.4	(R & H, 1974)
Tifton, GA (24)	-	2,324	128.1	136.9	0.95	52.7	10.5	17.3	(R & H, 1974)
Tifton, GA (24)	-	2,337	130.0	135.9	0.96	57.6	11.2	17.3	(R & H, 1974)
Fulwood, GA (24)	-	2,288	129.2	135.0	0.96	53.6	11.3	17.0	(R & H, 1974)
Range		2,096-2,589	107-142	112-156	0.79-1.10	40.6-68.6	7.0-12.7	16.1-18.8	
Range from	Table 4-1	2,047-2,321	115-129	115-142	0.88-1.03	50.3-56.3	7.9-8.2	16.3-18.1	

a = Total body length per body width, b = Total body length per length of esophagus, and c =Total body length per tail length.

[a] Number of nematodes tested.
[b] Mean and standard error.
[c] Missing data.
[d] Abu-Gharbieh and Perry (1970)
[e] Robbins and Hirshmann (1974)

Discussion

Morphological variation in a species is influenced by environmental conditions such as nutrient availability, and age structure of the population (Duncan et al., 1998). To eliminate some of these factors, all isolates of *B. longicaudatus* were cultured *in vitro* with the same host and under the same temperature.

Variation of *B. longicaudatus* morphology was detected. The body length of *B. longicaudatus* exhibited allometry with other characters because both LA and NC isolates had longer body length with longer tail length and excretory pore position than other isolates. On the other hand, the GV isolate had a shorter body length with shorter tail length and excretory pore position than other isolates. One of the differences between LA and NC isolates was the longer stylet length for the LA isolate, which also was confirmed in stylet-tail ratio and body length-stylet ratio. The LA, GA, and GV isolates had longer stylet than tail lengths with more than 1.0 for the stylet-tail ratio.

The morphometric variation of *B. longicaudatus* has been studied among populations (Abu-Gharbieh and Perry, 1970; Duncan et al., 1996; Robbins and Hirshmann, 1974) and within populations (Duncan et al., 1996). Data shown in Table 4-4 indicates a wide range of morphological variability of *B. longicaudatus*. One of the significant characters to differentiate among populations of *B. longicaudatus* was stylet length. The Georgia isolates were different from the North Carolina isolates with a longer stylet length. The central Florida isolates, especially from northeast Polk county, also was distinguished from other Florida isolates by having a longer stylet length.

However, the stylet-tail ratio was more than 1.0 for the central Florida isolates, but not for the Georgia isolates. The body length of the central Florida isolates was longer than other isolates, whereas the Gainesville, FL isolate had the shortest body length among all isolates. Compared to the five isolates of *B. longicaudatus* in this study, there are some similarities and dissimilarities in morphometric data. The style length of the LA isolate (our work) and the central Florida isolates (Duncan et al., 1996) was longer than other Florida isolates. However, mean values of the body length of the NC isolate and stylet length of the GA isolate (our work) were not as close as those of the North Carolina and Georgia isolates (Robbins and Hirshmann, 1974).

Robbins and Hirshmann (1974) compared the morphological variation in the stylet knobs and female vulva area between the Georgia and North Carolina isolates. Based on their report, the stylet knobs of the North Carolina nematodes are typically teardrop or kidney-shaped, whereas those of the Georgia isolate are typically rounded or oval. When the isolates reported herein were compared it was found that the stylet knobs of the GV, HA, LA, and NC isolates were kidney-shaped, but that of the GA isolate was oval-shaped. The latter finding is consistent with the report by Robbins and Hirshmann (1974). When the vulva area was studied among the isolates reported herein was found that the NC isolate had opposed sclerotized vaginal pieces that are lacking or very faint in the GA isolate. This result is opposite that reported by Robbins and Hirshmann (1974). The GV, HA, LA, and NC isolates possessed opposed vaginal pieces and there were morphological variations in vaginal pieces among them.

Belonolaimus longicaudatus is a polymorphic species. Morphological variation is often affected by environmental conditions. However, when isolates of *B. longicaudatus* were tested under the same host and temperature, variation was observed in stylet and body length among the five isolates of *B. longicaudatus*. In addition, variation in the morphology of stylet knobs and vaginal pieces was observed in the Georgia isolate. A great number of measurements from many different isolates of *B. longicaudatus* will be required to make clear the range of variation within and among isolates of *B. longicaudatus*.

CHAPTER 5
CHARACTERIZATION OF HOST SPECIFICITY IN *BELONOLAIMUS LONGICAUDATUS*

Introduction

Belonolaimus longicaudatus is an important ectoparasitic nematode that causes economic damage to many varieties of agricultural crops ranging from vegetables and grasses to ornamental and fruit trees (Cooper et al., 1959; Graham and Holdman, 1953; Perry and Rhoades, 1982). The variation among different populations of *B. longicaudatus* was noted in host specificity as well as morphology, and in some populations, variation in host specificity or host range was determined to be greater than morphological variation (Abu-Gharbieh and Perry, 1970; Robbins and Barker, 1973).

According to Robbins and Barker (1973), three North Carolina isolates of *B. longicaudatus* were clearly differentiated from a Tifton, Georgia isolate. The Georgia isolate had larger host range than the NC isolates. They also found that the reproductive rate of the Georgia isolate of *B. longicaudatus* was significantly greater than that of the North Carolina isolates. Three morphologically similar Florida isolates of *B. longicaudatus* also responded differently to different plants (Abu-Gharbieh and Perry, 1970).

The objective of this study was to determine whether there were differences is reproductive capabilities on different hosts of five isolates *B. longicaudatus* from Florida,

Georgia, and North Carolina. The experiment was designed to include the original host for each isolate and the reproductive rates were compared among the isolates.

Materials and Methods

Nematode Isolates and Greenhouse Culture of Nematodes

The *B. longicaudatus* isolates and the greenhouse cultures were the same as described in chapter 3.

Soils

The soil used in the experiment was collected from the University of Florida Green Acres agronomy farm located near Gainesville, FL. The soil was sieved and steam pasteurized. Soil texture was determined by the sodium metaphosphate-hydrometer procedure (Bouyoucos, 1936), and the composition was 95.5% sand, 2.0% silt, and 2.5% of clay. The plants were fertilized once a week with a 20-20-20 N-P-K solute fertilizer, and watered every day.

Host Plants

Peanut (*Arachis hypogaea* L. cv. Florunner), Cotton (*Gossypium hirsutum* L. cv. Sure grow 125), bermudagrass (*Cynodon dactylon* (L.) Pers.), citrus (*Citrus aurantium* cv. Sour orange), and potato (*Solanum tuberosum* L. cv. Sebago) were used to test for the host specificity of the five isolates of *B. longicaudatus*. The selected hosts except for peanut represented the crops from which the isolates were obtained. The experimental design was a completely randomized block and each treatment except cotton and potato was replicated five times. Cotton and potato were replicated four times.

For peanut and cotton, seeds were germinated under a moist paper towel for 3 days and for bermudagrass, nodal, 3 to 4 cm- long, stem cuttings of healthy bermudagrass were used. Seedlings of peanut and cotton, and stems of bermudagrass were individually transplanted into 20 cm-diam. clay pots filled with 1,200 ml pasteurized soil. Seventeen-days later, the soil was inoculated with 40 female and 20 male nematodes of each isolate extracted by the Baermann method. The roots and stems of peanut and cotton were collected after 75 days, and bermudagrass roots were collected after 67 days.

Two and half month old citrus seedlings were obtained from the University of Florida, Citrus Research and Education Center, Lake Alfred, FL, and they were transplanted into 20 cm-diam.-clay pots filled with 1,200 ml pasteurized soil. The five isolates of *B. longicaudatus* were inoculated 14 days later into the rhizosphere of 8 to 12 cm long citrus seedlings. For nematode inoculations, three 6 cm-deep holes were made approximately 3 cm apart from the base of plant stems. Sixty females and 20 males were added per pot. After 5 months, citrus roots and stems were collected.

Stem cuttings from greenhouse plants of potato were selected and grown for 19 days. Plants were transplanted to 20 cm-diam.-clay pots and artificially inoculated 7 days later with the five *B. longicaudatus* isolates. Each pot was inoculated with 40 females and 20 males. After 67 days, potato roots and tubers were collected.

Nematodes were extracted from 100 ml soil from each pot by a centrifugal flotation method (Jenkins, 1964), and the final number of nematodes was determined by counting under a stereomicroscope. The reproductive rate was calculated by Pf/Pi (final number of nematodes/initial number of nematodes) and significant differences among the five isolates were compared by Duncan's multiple-range test.

At the conclusion of each crop cycle the tops of plant was cut at the soil line, weight and dried in an oven at 70 °C for 3 days. Roots were also collected, weighted, and dried in an oven at 70 °C for 3 days.

Results and Discussion

There were no differences in the wet and dry weight of the plant tops or roots among the isolates tested (data not shown). All isolates of *B. longicaudatus* showed different reproductive capacity on the five different host plants. Both the GA and LA isolates reproduced well on cotton, bermudagrass, and citrus (Table 5-1). On the other hand, the NC and GV isolates reproduced poorly on all crops. The HA isolate reproduced well on cotton and potato.

The HA isolate showed greater host specificity to its original host of potato. The northeastern region of Florida, including Hastings, has a large potato growing area where *B. longicaudatus* is commomly found (Brodie, 1998). The economic threshold densities for the Hastings isolate were estimated at 2 to 3 *B. longicaudatus*/130 cm^3 of soil, and high Pi causes severe yield losses on potato (Crow et al., 2000). Potato was an excellent host for both North Carolina and Georgia isolates of *B. longicaudatus*, but the Georgia isolate showed approximately three times higher reproduction rate than three North Carolina isolates in a greenhouse experiment (Robbins and Barker, 1973). In this study, however, the reproductive capacity of the HA isolate on potato (16.5) was significantly higher than that of the GV (1.5), LA (5.5), GA (2.8), and NC isolates (1.4) ($P \leq 0.05$).

Both the NC and GV isolates were differentiated from other isolates by lower numbers of nematodes produced on cotton ($P \leq 0.05$). The host status of cotton for *B.*

longicaudatus has varied depending on different cultivars of cotton. For example 'Stoneville 7A' was a poor host for *B. longicaudatus* isolates from North Carolina and Georgia (Robbins and Hirshmann, 1973), whereas 'Coker 100WR' was a good host for an isolate from South Carolina (Graham and Holdeman, 1953). Crow et al. (2000) reported that the cultivar 'DPL 5415' was a good host for the Hastings isolate of *B. longicaudatus*.

In a host range test of Georgia and North Carolina isolates of *B. longicaudatus*, Robbins and Barker (1973) found that the Georgia isolate increased quickly with a higher reproductive rate than the North Carolina isolates. Abu-Gharbieh and Perry (1970) reported that Fuller's Crossing, Gainesville, and Sanford isolates of *B. longicaudatus* had different host specificities and reproductive rates on rough lemon, tomato, strawberry, and peanut. Conflicting results appeard in the following studies. A Georgia isolates did not reproduced on peanut (Brodie et al., 1970; Good, 1968), whereas Georgia, North Carolina, and Florida isolates reproduced on peanut (Abu-Gharbieh and Perry, 1970; Robbins and Barker, 1973). In this study, however, all isolates of *B. longicaudatus* reproduced poorly on peanut.

Table 5-1. Comparisons of the reproductive rate (the final number of nematodes/the initial number of nematodes) among five isolates[a] of *Belonolaimus longicaudatus* grown on different hosts.

Host plants	GV	HA	LA	GA	NC
	Pf/Pi (range)				
Peanut (*Arachis hypogaea* L. cv. Florunner)	4.4 ab (3.0-7.6)	3.2 b (2.2-4.4)	4.8 ab (2.4-6.8)	5.7 a (4.8-7.4)	1.1 c (0.2-2.0)
Cotton (*Gossypium hirsutum* L. cv. Sure grow 125)	26.8 b (25-31)	75.0 a (45-95)	77.0 a (58-118)	77.4 a (44-122)	29.3 b (23-37)
Bermudagrass (*Cynodon dactylon* (L.) Pers.)	6.2 c (2.2-12.6)	10.1 bc (7.0-15.8)	39.2 ab (4.0-101)	45.4 a (16.0-87.0)	11.3 bc (4.2-32.4)
Citrus (*Citrus aurantium* cv. Sour orange)	3.9 ab (0.5-9.8)	3.6 ab (0-9.5)	13.6 a (0-34.4)	8.5 ab (1.4-18.0)	0.2 b (0-0.5)
Potato (*Solanum tuberosum* L. cv. Sebago)	1.5 b (0-2.6)	16.5 a (2.2-31.4)	5.5 b (2.0-12.2)	2.8 b (1.8-3.8)	1.4 b (0-2.6)

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

[a] The isolates of *B. longicaudatus* were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), and corn in Scotland County, NC (NC).

CHAPTER 6
MOLECULAR CHARACTERIZATION OF *BELONOLAIMUS LONGICAUDATUS* ISOLATES BY SEQUENCE ANALYSIS OF ITS-1 rDNA

Introduction

Belonolaimus longicaudatus (Rau, 1958) is an economically important ectoparasitic nematode of crop plants. Its geographical distribution is limited to sandy soils (Brodie, 1976), but it is associated with a wide range of taxonomic groups of host plants including vegetables, grains, fruits, forage crops, turfgrasses, ornamentals, and forest trees (Christie, 1953; Esser, 1976; Holdeman, 1955; Robbins and Barker, 1973). The recent introduction of *B. longicaudatus* to California (Mundo-Ocampo et al., 1994), has increased its economic importance in agriculture.

The taxonomic status of *B. longicaudatus*, however, is unclear because of undefined variations in morphology and host specificity of different isolates. The first recognition of the variability of *B. longicaudatus* isolates was based upon different host pathogenicity among geographically different isolates (Good, 1968; Owens, 1951; Perry and Norden, 1963). Later, the morphology, host range, and hybridization tests were conducted to characterize Georgia and North Carolina isolates (Robbins and Barker, 1973; Robbins and Hirschmann, 1974). In Florida, morphologically similar isolates of *B. longicaudatus* showed differences in host specificity (Abu-Gharbieh and Perry, 1970). Based on these previous reports, the existence of possible races of the nematode must be

considered and additional studies to determine population and species characteristics of *B. longicaudatus* are desirable.

Modern molecular methods provide the potential to differentiate among morphologically similar isolates of nematodes to help elucidate their relatedness (Adams et al., 1998; Ferris et al., 1995; Gasse and Hoste, 1995; Zijlastra, 1997; Wendt et al., 1995; Zhu et al., 2000; Zijlastra et al., 1995). A molecular approach using PCR-RFLP was used to study *Belonolaimus* species systematics and distribution (Cherry et al., 1997). The screened target region of the DNA genome was the internal transcribed spacer (ITS-1) located between 18S and 5.8S in ribosomal DNA. Populations collected in the mid-western United States showed unique restriction profiles for the ITS-1 region (Cherry et al., 1997). The extent of heterogeneity of the ITS-1 DNA array indicates that concerted evolution (Dover, 1982) has not homogenized this region. Heterogeneity has been observed in other nematodes (Powers et al., 1997). However, the structural nature of heterogeneity of ITS-1 in *Belonolaimus* species is still unclear, and questions still remain about how much variation exists within individuals, and among populations. In addition, it is unknown whether the application of the ITS-1 sequence for phylogenetic analysis is reasonable and reliable.

Considering all the previous work on intraspecific variations of *B. longicaudatus*, ITS-1 was chosen to define the extent of diversity using a sequence already used to define phylogenetic relationship among eukaryotes. The sequence analysis of ITS-1 will provide information on genetic diversity and population structure of *B. longicaudatus*. In this study, therefore, seven different isolates of *B. longicaudatus* that were collected from different geographical locations and hosts were characterized by comparisons of their

ITS-1 sequences. The heterogeneity of ITS-1, and size variation of ITS-1 were examined within individuals, between isolates, and between species levels. The phylogenetic relationships among the different isolates of *B. longicaudatus* were analyzed by using different algorithms in PAUP (phylogenetic analysis using parsimony) program (Swofford, 1999). The objectives of the present study were to 1) examine ITS-1 sequence variations among different isolates of *B. longicaudatus*, 2) construct phylogenetic trees using ITS-1 sequence data, and 3) evaluate the utility of ITS-1 as a genetic marker to estimate the taxonomic status of isolates of *B. longicaudatus*.

Materials and Methods

Nematode Isolates

In addition to the five isolates of *B. longicaudatus* reported in chapters 3, 4, and 5, two additional isolates from Nebraska and Texas, *B. euthychilus,* and an unknown *Belonolaimus* sp. were used. The Nebraska isolate of *B. longicaudatus* was obtained from corn collected in Columbus, NE. The Texas isolate of *B. longicaudatus* was obtained from bermudagrass in Poteet, TX. The sequence of ITS-1 of a South Carolina isolate of *B. longicaudatus* was downloaded from Genebank (accession # U89696), and included in the data set for comparison.

As an out-group for comparison, *Belonolaimus euthychilus* was collected from roots of a pine tree located in The Natural Teaching Laboratory to the west of the Entomology & Nematology Building, Natural Area Drive, University of Florida, Gainesville. An unidentified *Belonolaimus* sp. that was collected from *Daphne* sp. in the Elizabeth garden located in Manteo, North Carolina was supplied by Dr. K. R. Barker.

Preparation of Nematode Sample

All *B. longicaudatus* were isolated directly from the original soil material. The nematodes from each isolate were extracted by the Baermann method (Ayoub, 1977), and picked by hand. Nematodes were placed in a 1.5 ml microcentrifuge tubes filled with sterile water and washed by centrifuging at 10,000*g* for 2 minutes three separate times.

DNA Extraction

Single female nematodes were hand picked, and placed on a glass cover slip in 10 µl sterile water (Powers and Harris, 1993) for the standard PCR, or in 25 µl sterile water for long PCR. Nematodes were cut into five or seven pieces with a sterile knife, and this material was directly mixed with PCR buffer, dNTP, primers, and sterile water for PCR amplification. Usually, four to five individual nematodes from each isolate were used for PCR amplification and ITS-1 products from a single female nematode were selected for further investigations.

PCR Amplification

Standard PCR. The DNA sample (10 µl) was suspended in a 25 µl reaction volume containing PCR buffer (10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl), 200 µM dATP, dGTP, dCTP, dTTP, 400 pM primers, and 0.8 units of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN) (Saiki, 1989). The upstream primer used was a 21-mer, 5'-TTGATTACGTCCCTGCCCTTT-3' (Vrain et al., 1997), located to include nucleotide positions from 199 to 178 upstream from the start of ITS-1. The downstream primer was a 20-mer, 5'-ACGAGCCGAGTGATCCACCG-3' (Cherry et al., 1997), which was homologous to the 3' end of 5.8S rDNA gene from the junction of ITS-

1. The DNA was amplified using three cycles. Each cycle consisted of a denaturation at 94 °C for 30 seconds, annealing at 56 °C for 1 minute, and extension at 72 °C for 1½ minutes. PCR products (10 µl) were analyzed by 1% agarose gel electrophoresis and 1 µl of fresh PCR product was used for cloning. The ITS-1 of GV, HA, LA, GA, NC, and TX isolates were amplified by the standard PCR method. A PERKIN–ELMER DNA Thermal Cycler 480 was used for all PCR assays.

Long PCR. DNA samples (25 µl) were mixed with 25 µl containing 50 mM Tris (pH 9.2), 16 mM ammonium sulfate, 1.75 mM $MgCl_2$, 350 µM dATP, dGTP, dCTP, dTTP, 800 pM of primers, 1 unit of *Pwo* and five units of *Taq* DNA polymerases (Barnes, 1994). Long PCR was performed using three linked profiles: 1) 1 cycle of denaturation at 94 °C for 2 minutes, 2) 10 cycles of denaturation at 94 °C for 10 seconds, annealing at 65 °C for 30 seconds, an extension at 68 °C for 1 minute, and 3) 25 cycles of denaturation 94 °C for 10 seconds, annealing at 65 °C for 30 seconds, plus an additional 20 seconds for every consecutive cycle. The primers used were a 30-mer, 5'-TTGATTACGTCCCTGCCCTTTGTACACACC-3' and a 30-mer, 5'-ACGAGCCGAGTGATCCACCGATGAGACTTG-3'. The primers were designed based on the sequences of ITS-1 of *B. longicaudatus* isolates amplified by the standard PCR method. Each 10 µl PCR sample was analyzed by 1% agarose gel electrophoresis and 1 µl of fresh PCR product was used for cloning. The ITS-1 of the NE isolate, *B. euthychilus* and the unidentified *Belonolaimus* sp. were capable of amplification by Long PCR method. A PERKIN–ELMER DNA Thermal Cycler 480 was used for all PCR assays.

Recombinant DNA Techniques

Recombinant DNA techniques, including restriction enzyme digestion, ligation, bacterial transformation, and plasmid DNA preparation were performed by standard methods (Sambrook et al., 1989). For the cloning of ITS-1, both Standard and Long PCR products were ligated into the vector plasmid pCR2.1-TOPO following the protocol provided by the manufacturer (Invitrogen Corporation, Carlbad, CA). The vector plasmid was transformed into *E. coli* cells upon mixing with β-mercaptoethanol (0.5 M). The transformed *E. coli* was mixed upon SOC medium (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.05% NaCl, 2 M $MgCl_2$, 20 mM glucose), and 100 µl of the mixture was spread on a petri plate containing Luria-Bertani (LB) agar, 50 µg/ml of ampicillin, X-gal and IPTG. The plates were incubated at 37 °C overnight. The clear transformed colonies were identified by their white color and 10 colonies were picked at random for analysis. For the analysis of positive clones, the selected colonies were cultured in 100 ml LB medium containing 50 µg/ml of ampicillin. After 16 hours of incubation, plasmids were extracted using QIAGEN Plasmid Midi-prep Kit (QIAGEN, Inc., Valencia, CA). The following protocol was used: 1) bacterial cells were harvested by centrifugation at 4,000 *g* for 10 minutes; 2) the bacterial pellet was re-suspended in buffer P1 (50 mM Tris Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A); 3) the P2 lysis buffer (200 mM NaOH, 1% SDS) was added gently, but thoroughly by inverting 4 to 6 times, and incubated at room temperature for 5 minutes; 4) chilled neutralization buffer P3 (3.0 M potassium acetate, pH 5.5) was added and incubated on ice for 5 minutes; 5) samples was centrifuged at 14,000*g* for 10 minutes and supernatant containing plasmid DNA removed; 6) samples were equlibrated with a QIAGEN-tip by applying buffer QBT (750 mM NaCl; 50 mM

MOPS, pH 7.0; 15% isopropanol), and the column allowed to empty by gravity flow; 7) supernatant was applied to the QIAGEN-tip and allow to enter the resin by gravity flow; 8) the QIAGEN-tip was washed with buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol); 9) DNA was eluted with buffer QF (1.25 M NaCl; 50 mM MOPS, pH 8.5; 15% isopropanol); 10) DNA was precipitated by adding iospropanol to the eluted DNA and centrifuged at 14,000*g* for 20 minutes; 11) the pellet was washed with 70% ethanol and centrifuge at 14,000*g* for 5 minutes; 12) the pellet was air-dried for 10 minutes and redissolved in the DNA in TE buffer (pH 8.0). The extracted pure plasmids were analyzed by restriction analysis (digested with *Eco*R I). After analysis of positive clones, both the strands of inserted DNA were sequenced using a PERKIN-ELMER applied Biosystem ABI PRISM Automated DNA Sequencer at the University of Florida ICBR (International Clinic of Biological Regeneration) Core Facility.

Sequence Alignment and Phylogenetic Analysis

All amplified DNA sequences were aligned after excluding the primer sequences by using the default parameters within CLUSTAL W (Thompson et al., 1994), and were then manually improved in the PAUP program (Swofford, 1999). All phylogenetic analyses were performed by using both distance and character based methods. The Kimura-2 parameter was selected for distance method, and maximum parsimony (MP), and maximum likelihood (ML) were used for character based methods. Through the heuristic search in PAUP 4.0 program (Swofford 1999), both MP and ML trees were constructed. The sequence variability within individuals and between isolates was determined by pair-wise comparisons in the PAUP program.

Results

The Size of ITS-1 in *Belonolaimus* Isolates

The sequenced ITS-1 with partial 18S and 5.8S segments resulted in some size variations among different isolates of *B. longicaudatus*. The sizes of total amplified DNA from all the Florida (GV, HA, LA), Georgia, and North Carolina isolates were identical at 706 bp (Fig. 6-1). However, the amplicons of the analogous region in the NB and SC isolates were 705 bp, and 704 bp, respectively (Table 6-1). The smallest size of the amplified region was found in the TX isolate with 664 bp, which was the result of a 41 bp deletion in the middle of ITS-1 region. The size of ITS-1 in the TX isolate (427 bp) was the smallest among all tested isolates of *B. longicaudatus*. Except for the TX (427 bp) and the NB (467 bp) isolates, all the other isolates showed exactly the same size for ITS-1 (468 bp).

Belonolaimus euthychilus and the unknown *Belonolaimus* sp. showed different profiles from *B. longicaudatus* in the size of the amplified DNA. The total amplified DNA of *B. euthychilus* and the unknown *Belonolaimus* sp. was approximately 600 bp (Fig. 6-2). There was also variation of ITS-1 size between two clones from each individual from *B. euthychilus* and the unknown *Belonolaimus* sp.. However, no size variation of ITS-1 was found between any two clones of the *B. longicaudatus* isolates (Table 6-1). In addition, the size of ITS-1 from *B. euthychilus* and the unknown *Belonolaimus* sp. were 361-363 bp and 359-364 bp, respectively, which was approximately 100 bp smaller than *B. longicaudatus*. Therefore, the heterogeneity of ITS-1 in terms of size occurred only in the out-group taxa, not within the in-group taxon of *B. longicaudatus*.

Heterogeneity of ITS-1 in Nucleotide

Heterogeneity of ITS-1in terms of nucleotide variation was observed within all isolates of *B. longicaudatus*, except for the TX isolate in which the two independent clones examined showed 100% similarity. The highest degree of dissimilarity was found in the LA isolate that had a 7 bp difference (4 bp transition, 3 bp transversion) between the two ITS-1 clones. Also, the NC isolate had a 4 bp transition and a 2 bp transversion, with a total 6 bp difference between the two ITS-1 clones. The GV and GA isolates had only a transition base pair difference, which was 1 bp and 3 bp difference, respectively. Therefore, the point mutation between ITS-1 copies was a common phenomenon in *B. longicaudatus* even though they were identical in size. In addition, the type of mutation in nucleotides appears to be varied among isolates with different ratios of transition to transversion.

Characterization of the Sequence of ITS-1

The total amplified DNA of *B. longicaudatus* that included part of 18S and 5.8S ribosomal gene segments and the entire ITS-1 region ranged from 664 bp to 706 bp (Fig. 6-3). The character numbers 1 to 177 is the end part of 18S, and 689 to 717 is the beginning part of 5.8S. Except for these two segments, the remaining portion (178 to 688) of ITS-1 ranged in length from 427 to 468 bp. Nucleotide mutation was common in the non-coding region of ITS-1, but was rare in the coding regions of the 18S or 5.8S. The out-group taxa, *B. euthychilus* and the unknown *Belonolaimus* sp., showed nucleotide variations in the same locations in the sequence encoding the 5' region of 18S rDNA which includes two transitions (G→A, and C→T) at 60 and 83, and one

transversion (A→T) at 64. However, *B. euthychilus* had an additional transversion (A→T) at 134. Even the in-group taxa, the NB and GA isolates showed transitions (G→A, and T→C) at 68 and 174 in 18S, respectively, and this nucleotide mutation occurred in the functional gene. Comparatively higher variations of the ITS-1 sequences were observed in the isolates of *B. longicaudatus*, whereas conservative nucleotide sequences were observed in both the 18S and 5.8S regions. The deletion of the nucleotides was observed in both the TX isolate and out-group taxa, and was formed between the position from 216 to 382 in the ITS-1 (Fig. 6-3). Within ITS-1, upstream from the start of the 5.8S gene, a unique repeated thymine (T) pattern was observed with a variable number of nucleotide substitutions, which also has been observed in the genera *Meloidogyne* (Zijlstra et al., 1995, 1997), and *Heterodera* (Szalanski et al., 1997).

Phylogenetic Analysis

Three different phylogenetic trees were constructed by using both distance and character based molecular phylogenetic algorithms. Bootstrap analysis detected values were above 50% for all *Belonolaimus* lineages determined by Neighbor-joining (NJ) (Fig. 6-4), Maximum parsimony (MP) (Fig. 6-5), and Maximum likelihood (ML) (Fig. 6-6) methods providing strong support for all of these trees. In addition, the general branch divergence patterns among the three trees were similar. The NB, SC, and TX isolates were clearly differentiated from the GA, HA, NC, LA, and GV isolates. In the NJ tree, the relationship between the NB and SC isolates were supported by a high bootstrap value of 99, which related to other *B. longicaudatus* isolates with an 84 bootstrap value. The TX isolate seems to have diverged more recently from the Florida, GA, and NC

isolates than the NB and SC isolates, which was supported by the bootstrap value of 100. This pattern was consistent in both ML and MP trees.

For FL, GA, and NC isolates, two separate copies of ITS-1 were sequenced to evaluate the heterogeneity problem in phylogenetic analysis. In the NJ tree (Fig. 6-4), two clones of ITS-1 from each isolate were supported as a monophyletic group with a bootstrap value more than 50, but the LA isolate was an exception. In the MP (Fig. 6-5) and ML (Fig. 6-6) trees, both the LA and GA isolates were not supported as a monophyletic group. The heterogeneity of ITS-1 in the LA isolate was too large and each copy of ITS-1 showed a different branch divergence in all three different trees. On the other hand, the GV isolate showed high similarity of nucleotide sequence between ITS-1 clones, which was supported by high bootstrap values in all three (ML, MP, and NJ) analysis methods.

The relationship among FL, GA, and NC isolates also was different between character-based and distance-based methods because of different bootstrap values. In both ML and MP, the relationship among the GA, HA, and NC isolates were poorly represented in tree topologies; however, based on the NJ tree, the GA, HA, and NC isolates were more closely related to each other than the LA and GV isolates. The GV isolate was assumed to be an ancestor group that diverged much earlier than the HA, LA, GA, and NC isolates, which was consistently implied in all three analysis methods.

Discussion

Variation in the size of the ITS-1 region was observed among the various isolates of *B. longicaudatus* as well as between different species of *Belonolaimus*. The genus

Belonolaimus had been categorized into a group of nematodes that have little or no size variation in their ITS-1 (Powers et al., 1997). However, approximately a 100 bp difference between *B. euthychilus* and *B. longicudatus* was determined in this study. The TX isolate of *B. longicaudatus* showed a 41 bp deletion in ITS-1, and exhibited a unique profile among the various isolates of *B. longicaudatus*. Furthermore, the ITS-1 size polymorphism was not only limited among species or isolates, but also within individual nematodes. For instance, both *B. euthychilus* and the unknown *Belonolaimus* sp. showed ITS-1 size heterogeneity within each individual nematode. However, ITS-1 size heterogeneity was not detected in any of the isolates of *B. longicaudatus*. Thus, size heterogeneity appears to be a characteristic limited to certain species of *Belonolaimus*. Based on nucleotide variation of the ITS-1, the putative ITS-1 heterogeneity of *Belonolaimus* was already inferred by the profiles of the PCR-RFLP (Cherry et al., 1997). However, the detailed information here provides a more rigorous comparison with which to evaluate phylogeny of *Belonolaimus* species.

The heterogeneity of ITS has been reported for free-living nematodes (Joyce et al., 1994), plant-parasitic nematodes (Szalanski et al., 1997; Zijlstra et al., 1995, 1997), certain insect species (Rich et al., 1997; Onyabe and Conn, 1999), and even in angiosperms (Baldwin et al., 1995). In nematodes, however, the characteristics of ITS-1 are seen as more species specific. For instance, there was no heterogeneity in the size of ITS-1 in *Heterorhabditis* (Joyce et al., 1994), *Heterodera*, *Globodera*, *Meloidogyne*, *Hoplolaimus*, *Trichodorus*, and *Xiphinema* (Powers et al., 1997), whereas size variation was detected *in Aphelenchoides* (Ibrahim et al., 1994), *Tylenchorhynchus* (Powers et al., 1997), and *Pratylenchus* (Orui, 1996). The heterogeneity of nucleotide variation in ITS-1

is commonly observed in most sequences from different species of plant-parasitic nematodes, which includes *Meloidogyne* (Zijlstra et al., 1995, 1997), *Heterodera* (Szalanski et al., 1997), *Belonolaimus* (Cherry et al., 1997), and *Globodera* (Thiery et al., 1996). The significance of ITS heterogeneity is being discussed because of questions concerning the suitability of sequences for phylogenetic analyses, and the problems related to the application of sequence data from ITS-1 (Rich et al., 1997; Onyabe and Conn, 1999).

The heterogeneity of the LA isolate failed to group two sequences of ITS-1 into a monophyletic group as seen in the ML, MP, and NJ trees. The GA isolate also failed in two clones of ITS-1 sequences to fall into a single phyletic group because of ITS-1 heterogeneity (ML and MP trees). However, the tree topologies given by different phylogenetic analyses were similar to each other, and form a basis for understanding the relationship among the isolates. In addition, the sequence data of ITS-1 itself is useful information to compare and characterize the different isolates of *Belonolaimus*.

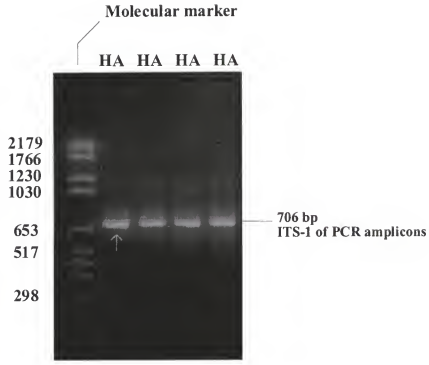


Figure 6-1. PCR amplification of ITS-1 by standard PCR loaded on 1% agarose gel. The nematode sample used was a single female from the Hastings isolate and each line showed ITS-1 of PCR amplicons from a single female. Arrow indicates the ITS-1 gene product used for the cloning procedure.

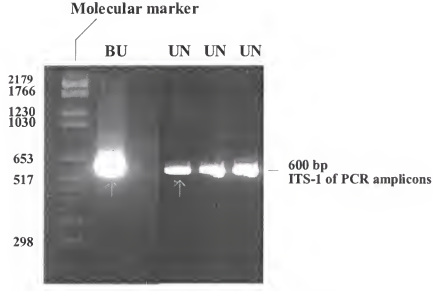


Figure 6-2. PCR amplification of ITS-1 by long PCR loaded on 1% agarose gel. The nematode sample used was a single female from the *Belonolaimus euthychilus* (BU) and an unidentified *Belonolaimus sp.* (UN). Each line showed ITS-1 of PCR amplicons from a single female. Arrows indicates the ITS-1 gene product used for the cloning procedure.

```
          10        20        30        40        50
          .         .         .         .         .
GA-1  GTACACACCGCCCGTCGCTGCCCGGGACTGAGCCATTTCGAGAAACTTGG
GA-2  ..................................................
LA-1  ..................................................
LA-2  ..................................................
GV-1  ..................................................
GV-2  ..................................................
HA-1  ..................................................
HA-2  ..................................................
NC-1  ..................................................
NC-2  ..................................................
TX    ..................................................
NB    ..................................................
SC    ..................................................
BU    ..................................................
UN    ..................................................
```

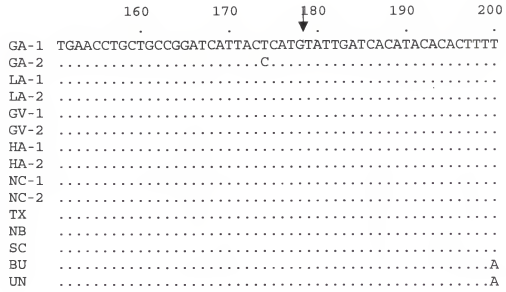
Figure 6-3. Multiple sequence alignment of ITS-1of in-group *Belonolaimus longicaudatus* isolates, and out-group *Belonolaimus euthychilus* and unknown *Belonolaimus* sp. including partial 18S (1-177) and 5.8S (689- 717). Isolates of *Belonolaimus longicaudatus* were obtained from cotton in Tifton, Georgia (GA), from citrus in Lake Alfred, Florida (LA), from bermudagrass in Gainesville, Florida (GV), from Potato in Hastings, Florida (HA), from corn in Scotland County, North Carolina (NC), from corn in Poteet, Texas (TX), and from corn, Columbus, Nebraska (NB). *Belonolaimus euthychilus* isolate was collected from a pine tree in Gainesville, Florida (BU) and the unidentified *Belonolaimus* sp. was obtained from host, *Daphne* sp. in Manteo, North Carolina (UN). The sequence of SC (South Carolina) isolate was downloaded from the Genebank (accession # U89696). The numbers (1 and 2) for GA, LA, GV, HA, and NC isolates represent two different sequences of ITS-1from two clones of a single female nematode for each isolate. Dots indicate nucleotide identity, and hyphens indicate gaps. Arrows indicates the nucleotide position of the start (178) and end (688) of ITS-1.

Figure 6-3. Continued.

```
                60         70         80         90         100
                 .          .          .          .          .
GA-1  GGATTGTTGGTTTAGCGGTTTTCGGATCGCTTCATCGATGAGAACCAATT
GA-2  ..................................................
LA-1  ..................................................
LA-2  ..................................................
GV-1  ..................................................
GV-2  ..................................................
HA-1  ..................................................
HA-2  ..................................................
NC-1  ..................................................
NC-2  ..................................................
TX    ..................................................
NB    ................A.................................
SC    ..................................................
BU    .........A...T..................T.................
UN    .........A...T..................T.................
```

```
               110        120        130        140        150
                 .          .          .          .          .
GA-1  TAATCGCAGTGGCTTGAACCGGGCAAAAGTCGTAACAAGGTGGCTGTAGG
GA-2  ..................................................
LA-1  ..................................................
LA-2  ..................................................
GV-1  ..................................................
GV-2  ..................................................
HA-1  ..................................................
HA-2  ..................................................
NC-1  ..................................................
NC-2  ..................................................
TX    ..................................................
NB    ..................................................
SC    ..................................................
BU    ..................................................
UN    ..............................T...................
```

Figure 6-3. Continued.



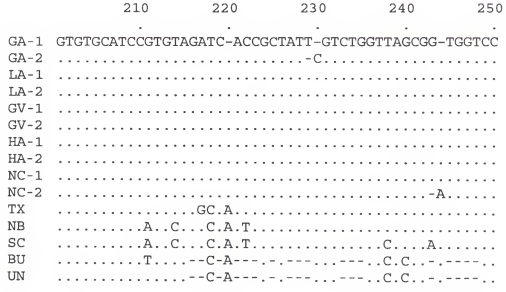


Figure 6-3. Continued.

```
              260        270        280        290        300
               .          .          .          .          .
GA-1  GATGAAGTAGCG-TATGCATCTGGC--TTGTCTGATGGGCTAGCTTTTCG
GA-2  ....................................................
LA-1  ....................................................
LA-2  ...........-A...........-T..........................
GV-1  ..................................T.................
GV-2  ..................................T.................
HA-1  ..........................................G.........
HA-2  ..........................................G.........
NC-1  ....................................................
NC-2  ........................G-..........................
TX    ......A....A..C-A-.A................C--------C--.
NB    ...T.........C---.GT..............C.T.TAA.GC.-.
SC    ...T.........C---.G........C..........T.T.A.GC.-.
BU    -..T--.------..C---.GT...-..----.-----.--T.--------.
UN    -..T--.------..C---.GT...-..----.-----.--T.--------.
```

```
              310        320        330        340        350
               .          .          .          .          .
GA-1  GCCACTAAGCCTAAT-GTGG-CT-GACTCTAGC-CC-GCTG-GTTGCATG
GA-2  ..................................-T..............
LA-1  ..............-A....T-..............................
LA-2  ....................................................
GV-1  ....................................................
GV-2  ....................................................
HA-1  ..............................-T..................
HA-2  ..............................-T..................
NC-1  ......................-A..................-A.......
NC-2  ..............................-T..................
TX    --------GT-.---...--.--.C----T---.--...-.--------
NB    .........---.T..T........T..CGTGA.CA..............T...
SC    .........---.T..G........T.A-GTGA.CA..............T...
BU    ----------G.---.-.-.--.-------A-.--.-.--.--------
UN    ----------G.---.-.-.--.-------A-.--.-.--.--------
```

Figure 6-3. Continued.

```
            360        370        380        390        400
             .          .          .          .          .
GA-1 TTATGGAGTCGACGCTTGTCTGGTA-GCGTTGCACGGACCGTGCCCGTGA
GA-2 ..........................................................
LA-1 ..........................................................
LA-2 ..........................................................
GV-1 ..........................................................
GV-2 ..........................................................
HA-1 ..........................................................
HA-2 ..........................................................
NC-1 ..........................................................
NC-2 ..........................................................
TX   --.........T.........A..A..A.....T.............
NB   C.....................A..........T....T.........
SC   C.....................A........A..T.............
BU   ---.------...A-----..----------..T.............
UN   ---.------...A-----..----------..T.............
```

```
            410        420        430        440        450
             .          .          .          .          .
GA-1 TTTGGTCATTGTCCG--TTGACCCGTTGTGCTGGATGGTGT---CTGTCT
GA-2 ..........................................................
LA-1 ..........................................................
LA-2 ..........................................................
GV-1 ..........................................................
GV-2 ..........................................................
HA-1 ..........................................................
HA-2 ..........................................................
NC-1 ..........................................................
NC-2 ..........................................................
TX   .C..........T.G.............T..........TGT......
NB   .C..........T.G..........T..T..........TGC......
SC   .C..........T..A.....T.......T..........TGT....T.
BU   .C.........A.TCTA........C....T..........TTTG.TC..
UN   .C.........A.TCTA........C....T..........TTTG.TC..
```

Figure 6-3. Continued.

```
            460        470        480        490        500
             .          .          .          .          .
GA-1  G-GAACGCCAGTCC--AGTTCGGCGTTCATAGGGCCTAACGGCTTTGCTG
GA-2  ...........................................
LA-1  ...........................................
LA-2  ............-A.......A.....................
GV-1  ..T........................................
GV-2  ..T........................................
HA-1  ...........................................
HA-2  ............-.T.............................
NC-1  ...........................................
NC-2  ...........................................
TX    .CAG-............GT.......C..................
NB    .CAG-.......A.....C.T.A......C..............
SC    ACAG-....GA.......CGT.A....T.C..............
BU    AAA--.......TT....CAT..T.....C..............
UN    AAA--.......TT....CAT..T.....C..............
```

```
            510        520        530        540        550
             .          .          .          .          .
GA-1  GCGTCTATGCGTGGTTGAGCAGTTGTTGTCTCTCCGTCCGTGGCTGTGAT
GA-2  ...........................................
LA-1  ...........................................
LA-2  ...........................................
GV-1  ...........................................
GV-2  ...........................................
HA-1  ...........................................
HA-2  ...........................................
NC-1  ...........................................
NC-2  ...........................................
TX    ...........................................
NB    ................T..........................
SC    ................T..........................
BU    ......T.....................C....T.....C...
UN    ......T.....................C....T.....C...
```

Figure 6-3. Continued

```
            560        570        580        590        600
             .          .          .          .          .
GA-1 GAGACCGCGCGTTAGGGCCCATGCCTTGCCTCTGGCATGTTGGCTTA-AG
GA-2 ..........................................................
LA-1 ..........................................................
LA-2 ..........................................................
GV-1 ..........................................................
GV-2 ..........................................................
HA-1 ..........................................................
HA-2 ..........................................................-G..
NC-1 ..........................................................
NC-2 ..........................................................
TX   ....................T.....................................
NB   ....................T.................A.....................
SC   ....................T.....................................
BU   .....T.....................................................
UN   .....T..................T.....................................
```

```
            610        620        630        640        650
             .          .          .          .          .
GA-1 ACTTGATGAGCGCGATGCCTT-GCGCCGCCAGTACCCACT-TTTTT---C
GA-2 ..........................................................
LA-1 ..........................................-A..........
LA-2 ..........................................................
GV-1 ..........................................................
GV-2 ..........................................................
HA-1 ..........................................................
HA-2 ....................-C..................................
NC-1 ..........................................................
NC-2 ..........................................................
TX   ...............................C............T...
NB   ...............................C..............
SC   ...............................C..........A.....
BU   ..............T.................C...A.........T...
UN   ..............T.................C...A.........TTT.
```

Figure 6-3. Continued

```
                660        670        680        690        700
                 .          .          .          .          .
GA-1  ATTA-TTTTTTTTG-TTTGAAGCAA--A-GCAAATTCAAGTCTCATCGG
GA-2  ..........................................................
LA-1  ....T-....................................................
LA-2  .....C....................................................
GV-1  .....C....................................................
GV-2  .....C....................................................
HA-1  ..........................................................
HA-2  ......................A...................................
NC-1  ..............-A........................................
NC-2  ..........................................................
TX    .....C............C....T--.G.A.-..........................
NB    .C...CA..........C.....--.G.A.-..........................
SC    .C...CA...A.A.....C.....--.G.A.-..-.......................
BU    .C..T--.......A...CAT.TTG.TG.A-..........................
UN    .C..T--......-A...CAC.TTG.TG.A-..........................
```

```
                710
                 .
GA-1  TGGATCACTCGGCTCGT
GA-2  .................
LA-1  .................
LA-2  .................
GV-1  .................
GV-2  .................
HA-1  .................
HA-2  .................
NC-1  .................
NC-2  .................
TX    .................
NB    .................
SC    .................
BU    .................
UN    .................
```

Table 6-1. The size variations of total amplified DNA, and ITS-1 products from different isolates of *Belonolaimus longicaudatus*, *Belonolaimus euthychilus*, and an unknown *Belonolaimus* sp.

Clone	Geographical origin	Host plant	Species	ITS-1 length (bp)	Total amplified DNA (bp)
pHR 1	Tifton, GA	Cotton	*B. longicaudatus*	468	706
pHR 2	Tifton, GA	Cotton	*B. longicaudatus*	468	706
pHR 3	Scotland County, NC	Corn	*B. longicaudatus*	468	706
pHR 4	Scotland County, NC	Corn	*B. longicaudatus*	468	706
pHR 5	Hastings, FL	Potato	*B. longicaudatus*	468	706
pHR 6	Hastings, FL	Potato	*B. longicaudatus*	468	706
pHR 7	Lake Alfred, FL	Citrus	*B. longicaudatus*	468	706
pHR 8	Lake Alfred, FL	Citrus	*B. longicaudatus*	468	706
pHR 11	Gainesville, FL	Bermudagrass	*B. longicaudatus*	468	706
pHR 12	Gainesville, FL	Bermudagrass	*B. longicaudatus*	468	706
pHR 22	Columbus, NB	Corn	*B. longicaudatus*	467	705
U89696[a]	South Carolina	ND[b]	*B. longicaudatus*	468	704
pHR 13	Poteet, TX	Bermudagrass	*B. longicaudatus*	427	664
pHR 14	Poteet, TX	Bermudagrass	*B. longicaudatus*	427	664
pHR 17	Gainesville, FL	Pine tree	*B. euthychilus*	363	603
pHR 18	Gainesville, FL	Pine tree	*B. euthychilus*	361	601
pHR 19	Manteo, NC	*Daphne* sp.	*Belonolaimus sp.*	364	604
pHR 20	Manteo, NC	*Daphne* sp.	*Belonolaimus sp.*	359	599

[a] The genebank accession number for South Carolina isolate of *B. longicaudatus*.

[b] The original host for the South Carolina isolate was not determined.

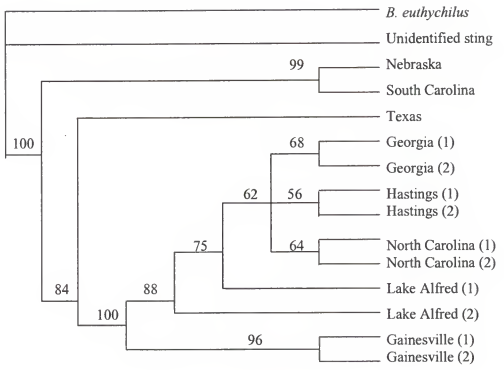


Figure 6-4. Neighbor-joining tree based on ITS-1 sequence data of *Belonolaimus longicaudatus* isolates, *Belonolaimus euthychilis*, and an unknown *Belonolaimus* sp. The tree was constructed by using the distance method of Kimura's 2-parameter. Bootstrap values in each branch line represent the supported numbers per 100 replications.

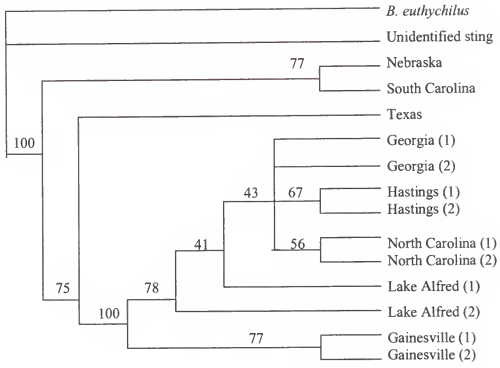


Figure 6-5. Parsimony tree based on ITS-1 sequence data of *Belonolaimus longicaudatus* isolates, *Belonolaimus euthychilis*, and an unknown *Belonolaimus* sp. The tree was constructed by using the optimality criterion of maximum parsimony method. Bootstrap values in each branch line represent the supported numbers per 100 replications.

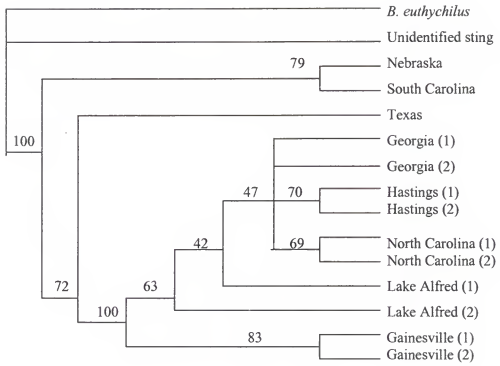


Figure 6-6. Maximum likelihood tree based on ITS-1 sequence data of *Belonolaimus longicaudatus* isolates, *Belonolaimus euthychilis*, and an unknown *Belonolaimus* sp. The tree was constructed by using the optimality criterion of maximum likelihood method. Bootstrap values in each branch line represent the supported numbers per 100 replications.

CHAPTER 7
SUMMARY

Five isolates of *Belonolaimus longicaudatus*, from different hosts and geographical locations, were tested to determine their intraspecific variations. Variation among different isolates of *B. longicaudatus* was recognized in the early 1950's when they were compared on different hosts. Some researchers suggested the existence of physiological races within *B. longicaudatus* and others even suggested multiple species.

The objectives of this research were to 1) determine the variation level in biology, morphology, and host specificity among isolates of *B. longicaudatus*, 2) determine the variation level of the sequences of ITS-1 in rDNA among different isolates, and 3) examine the utility of ITS-1 of rDNA as a genetic marker for differentiation of *B. longicaudatus* isolates based on phylogenetic analyses. Therefore, this thesis was designed as an integrated approach using both classical taxonomy methods and advanced molecular phylogenetic analyses.

The biology of *B. longicaudatus* is not well defined. With the advent of artificial cultivation of the nematode it is now possible to observe biological characters during development. The timing of each development stage of the different isolates of *B. longicaudatus* was compared by culturing all isolates on excised corn roots grown on Gamborg B-5 medium (Huettel and Rebois, 1985). The developmental stages were tracked from egg to adult. The NC isolate required 25 days for completing its development, which was the longest period for any isolate ($P \leq 0.05$). The second

longest period was 22.9 days for the LA isolate ($P \leq 0.05$). Among the GV, HA, and GA isolates, there were no differences among developmental times with each lasting 19.5, 19.2, and 18.1 days, respectively. The NC isolate had a longer egg and J4 developmental period than others isolates ($P \leq 0.05$). Both the NC and LA isolates had longer J2 and J3 periods than those of the GV, HA, and GA isolates. The time required for molting was consistent in each developmental stage, but the fourth molt took longer than the second and third molts. There was no significant difference in molting times for any of the different isolates of *B. longicaudatus*. The range of variation was greater in egg and J4 developmental periods than for J2 and J3 periods. The J3 period had the smallest variation in developmental time among all stages.

Variation among morphological characters was used to differentiate among females of five different isolates of *B. longicaudatus*. The LA and NC isolates were characterized by longer body length, greater body width, head to excretory pore distance, and tail length. The LA isolate was distinctive in having the longest stylet (stylet cone, shaft, and knobs), and the highest head constriction among all isolates ($P \leq 0.05$). On the other hand, the GV isolate was smaller in body length, head to excretory pore distance, and stylet length (stylet cone, shaft, and knobs). In stylet-tail ratio, the GA, LA, and GV isolates showed longer stylet than tail ratio, whereas the NC and HA isolates had a longer tail than stylet. The HA isolate showed vulva position with the greatest anterior location compared to that of other isolates. In other morphological characters, the stylet knobs of the GV, HA, LA, and NC isolates were kidney-shaped, but those of the GA isolate were oval-shaped. However, in the vulva area, the NC isolate had opposed sclerotized vaginal pieces that are lacking or very faint in the GA isolate, which was a contradictory

observation from a previous report. The vaginal pieces of the LA isolate were most prominent and clearly recognized among all isolates. The HA and GV isolates possessed smaller vaginal pieces than others, and those of the NC isolate were weakly developed and not clearly recognizable.

The J2 from five isolates showed different characteristics in body length, body width, stylet length (both stylet cone and stylet shaft and knobs), and head height. The LA isolate was larger in stylet length (both stylet cone, shaft, and knobs) and head height, whereas the GV isolate had a shorter stylet length compared with the other isolates ($P \leq 0.05$). In body length and body width, the NC isolate had the longest body and the largest body width, however, the GV isolate had the shortest body and smallest body width among all isolates ($P \leq 0.05$). The HA isolate was distinctive with a lower head constriction ($P \leq 0.05$).

All isolates of *Belonolaimus longicaudatus* showed distinctive reactions to five different host plants of cotton, citrus, peanut, bermudagrass, and potato. Both the GA and LA isolates reproduced well on cotton, bermudagrass, and citrus. On the other hand, the NC and GV isolates reproduced poorly on all crops. The HA isolate reproduced well on cotton and potato. The HA isolate showed greater host specificity to its original host of potato whereas the GV isolate did not reproduce well on its original host of bermudagrass. Except for the HA, all isolates reproduced poorly on potato, which clearly differentiated the HA isolate from the others ($P \leq 0.05$). Both the NC and GV isolates were differentiated from other isolates by lower number of nematodes produced on cotton ($P \leq 0.05$). There was no difference in dry or fresh root-stem weight among all hosts.

For molecular phylogenetic characterization of *B. longicaudatus*, the ITS-1 in ribosomal DNA was cloned and sequenced. The size of total amplified DNA from all the Florida (HA, LA, GV), Georgia, and North Carolina isolates were identical with 706 bp but that of the Nebraska and South Carolina isolates were 705 bp and 704 bp, respectively. However, the smallest size of total DNA was found in the Texas isolate with 664 bp, which was caused by a 41 bp deletion in the middle of ITS-1 region. Therefore, the size of ITS-1 in the Texas isolate (427 bp) was the smallest among all those tested. Except for the Texas isolate (427 bp) and the Nebraska isolate (467 bp), all the other isolates were the same size at 468 bp.

Heterogeneity of ITS-1in terms of nucleotide variation was observed within all isolates of *B. longicaudatus*, except for the Texas isolate in which the two independent clones from a single isolate examined showed 100% similarity. The highest degree of dissimilarity was found in the LA isolate that had a 7 bp difference (4 bp transition, 3 bp transversion) between the two ITS-1 clones. Also, the NC isolate had a 4 bp transition and a 2 bp transversion, with a total 6 bp difference between the two clones (ITS-1). The GV and GA isolates had only a transition without a transversion, which were different by 1 bp and 3 bp, respectively. Therefore, the point mutation between ITS-1 copies appeared to be a common phenomenon in *B. longicaudatus* even though they were identical in size.

For phylogenetic analysis, three different phylogenetic trees were constructed by using both distance and character based molecular phylogenetic algorithms. Bootstrap analysis detected values above 50% for all *Belonolaimus* lineages in Maximum parsimony (MP), Neighbor-joining (NJ), and Maximum likelihood (ML) trees. In

addition, the general branch divergence patterns among the three trees were similar. The Nebraska, South Carolina, and Texas isolates were clearly differentiated from the Florida (GV, HA, LA), Georgia, and North Carolina isolates. In the NJ tree, the relationship between the Nebraska and South Carolina isolates were supported by a high bootstrap value of 99, which related to other *B. longicaudatus* isolates with an 84 bootstrap value. The Texas isolate seemed to have diverged more recently from the Florida, Gorgia, and North Carolina isolates than the Nebraska and South Carolina isolates, which was supported by a 100 bootstrap value. This pattern was consistent in both ML and MP trees.

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BIOGRAPHICAL SKETCH

I was born in Andong, Korea, on January of 1970. With two sisters and one brother, I was raised by dedicated parents Su-Mi Han, my father, and Jung-Sook Oh, my mother. In Andong I spent most of my childhood, and also finished my university education. I achieved my bachelor and MS degrees in the Agricultural biology department of Andong National University.

During my bachelor studies, I attained the general background of biology including plant pathology and entomology. For my MS, I decided to study a new area of Nematology and I studied the anatomical and biochemical characteristics of *Pratylenchus vulnus*. In 1995, I finished my MS program, and decided to do further study of plant parasitic nematodes in United States.

In summer of 1996, finally I entered University of Florida, and started the Ph.D program under Dr. Dickson and Dr. Weingartner. I am a member of the Gamma Sigma Delta Honor Society of Agriculture, and the Society of Nematology. During my Ph.D, I married with Sung-Ho Park, and now we have one lovely son, Ho-Jin Park.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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